DEXTRAN GELS AND THEIR APPLICATIONS IN GEL FILTRATION

BY

PER FLODIN

Dextran gels and their applications in gel filtration

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DEXTRAN GELS AND THEIR APPLICATIONS IN GEL FILTRATION

INAUGURAL DISSERTATION

BY

PER FLODIN
FIL. LIC., VG.

BY DUE PERMISSION OF THE FACULTY OF SCIENCE OF THE UNIVERSITY OF UPPSALA, TO BE PUBLICLY DISCUSSED IN THE LECTURE HALL OF THE INSTITUTE OF CHEMISTRY, MAY 16, 1962, AT 10 A. M. FOR THE DEGREE OF DOCTOR OF PHILOSOPHY. IN ADDITION TO THIS SURVEY THE PAPERS PORATH AND FLODIN (1959), FLODIN ET AL. (1960), FLODIN (1961), GRANATH AND FLODIN (1961), FLODIN AND ASPBERG (1961), AND FLODIN AND KILLANDER (1962) IN THE LIST OF REFERENCES WILL BE PUBLICLY DISCUSSED.

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Dextran gels and their applications in gel filtration

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To my Wife

Preface

As a result of a cooperative research programme carried out jointly at the Institute of Biochemistry and AB Pharmacia, Uppsala, dextran gels for separations on a molecular size basis were introduced a few years ago. Since then the gels have found numerous applications and many publications concerning them have appeared. This book was prepared with the intention of presenting an overall picture of the present status of the preparation, properties, and applications of the dextran gels. Unpublished experiments are presented and many publications from this and other laboratories are quoted. The work reported in chapter 10 was made in collaboration with Mr Arne Emnéus.

I wish to express my gratitude to Prof. Arne Tiselius for his stimulating interest and helpful advice. I am greatly indebted to Mr Elis Göth, president of AB Pharmacia, for his encouragement, interest and support throughout the progress of this work. To my collaborators, Dr Björn Ingelman, Dr Jerker Porath, Dr Bertil Gelotte, Dr Kirsti Granath, Mr Arne Emnéus, and Dr Johan Killander as well as to the members of the staffs of AB Pharmacia and the Institute of Biochemistry I express my gratitude for kind help and stimulating discussions.

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Uppsala, March, 1962.

PER FLODIN

CHAPTER 1

Introduction

In 1861 Sir Thomas Graham introduced dialysis as a means of separating colloids from crystalloids. The method has subsequently become an indispensible tool, especially for biochemists. Proteins, nucleic acids, and many other macromolecules ocurring in nature are labile and thus a gentle method, like dialysis is of great value in their separation from low molecular weight substances. A large number of papers has been published on the theory and practice of dialysis and related methods, (cf. Stauffer, 1956). It will suffice here to mention the recent work of Craig and coworkers (1960), which has thrown new light on the mechanism and applications of the method. They determined the 50 per cent escape times of a number of solutes through cellophane membranes. The order of increasing escape times were found to be roughly the same as the order of increasing molecular weights (Craig et al., 1957). Chemical modification by which the membranes were rendered more hydrophobic and thus less porous resulted in increased escape times. The opposite effect was obtained when the membranes were subjected to mechanical stretching.

In collodion membranes synthetic amyloses varying in degree of polymerization have been fractionated (Mould and Synge, 1954). The water flow obtained by electroendoosmosis was utilized to transport the solutes through the membrane strips. The retardation was found to increase with increasing molecular weights. Experiments with membranes of varying pore size showed that the most porous ones admitted the largest molecules. It was concluded that collodion membranes acted as molecular sieves, though they did not possess the extreme selectivity of the zeolites.

In the 1920's a number of papers on the properties of zeolitic minerals appeared. They had the interesting property of sorbing small in preference to large, molecules and were given the name "molecular sieves". The fundamental work of Barrer has led to a thorough understanding of the structure and function of zeolites and it has also led to the use of synthetic zeolites on a technical scale. These are crystalline aluminosilicates with well defined porous structures (Hersh, 1961). The pore size is uniform throughout the crystals, which explains their extreme selectivity. Thus in the commercial types, Linde Molecular Sieves 4 A and 5 A, n-paraffins up to C_{14} are sorbed while isoparaffins are not sorbed due to their larger minimal dimensions. The very high affinity for water makes the zeolites useful for drying gases and organic liquids and has resulted in large -scale technical applications.

In ion exchange resins it has often been observed that the capacity for an ion depends on its size (Helfferich, 1959). The explanation is the discrimination induced by the three-dimensional matrix to which the fixed charges are connected. The capacity for large ions is increased by decreasing the number of cross-linkages whereby a larger porosity is obtained. The differential capacity of ion exchange resins for ions of different size can be utilized for separations. As examples can be mentioned the removal of lanthanum and beryllium ions from colloidal zirconium hydroxide (Ayres, 1947). In a similar way galacturonic acid was removed from solutions of pectin (Deuel et al., 1950). In separations of this type losses can occur due to absorption to the surface of the resin particles. Thus in the separation of amino acids from proteins the best recoveries were obtained with large particles and a moderate excess of resin (Partridge, 1952).

In passing nonelectrolytes through a bed of an ion exchange resin, effects are encountered which depend on the molecular sieving effect of the resin network. Wheaton and Bauman (1953) found that both sorption and size effects were responsible for the separations obtained in cross-linked polystyrene resins. In some cases, e.g. in the separation of glucose and methanol, molecular sieving apparently predominated. The sieving effect of Dowex 50-X12 was utilized for the analysis of mixtures of polyhydric alcohols (Clark, 1958). The resin in the hydrogen form resolved the mixture into three major fractions containing sorbitol, glycerol, and glycols. The solutes appeared in the effluent in the order of decreasing molecular weight.

In early studies on the use of water-swollen starch as a supporting material in zone electrophoresis in columns it was found that proteins and amino acids were eluted at different rates (Porath and Flodin, unpublished results). Similarly, a peptide mixture was divided into two fractions of differing molecular weights (Lindqvist and Storgårds, 1955). It was not until the publications of Lathe and Ruthven (1955 and 1956) appeared that the graded penetration into starch granules of solutes differing in size was described. They studied both nonelectrolytes and electrolytes and the former with few exceptions were found to be eluted in the order of their molecular weights. Many charged solutes, on the other hand, showed varying elution behaviour when eluted at different pH values. The largest discrepancies were observed for basic and aromatic compounds. In general, only solutes with molecular weights below 1,000 penetrated potato starch granules. When similar experiments were performed in columns packed with heat-swollen maize starch granules larger molecules, such as hemoglobin, inulin, and bacitracin A were retarded in relation to colloidal carbon.

The use of a granular cross-linked galactomannan for the separation of polysaccharides and salts was briefly mentioned in a paper by Deuel and Neukom (1954). Unaware of these results Porath and Flodin (1959) introduced particulate dextran gels as a means of separating solutes differing in size, e.g.

for desalting proteins and other macromolecules. The cross-linked dextran gels, which are commercially available under the trade name "Sephadex"[1], have been found valuable for the separation of water-soluble mixtures according to size. Many of these results will be referred to in later chapters. The chromatographic procedure in which the molecular sieve properties of gels are utilized has been called gel filtration. A review on molecular sieves in chromatography has appeared (Flodin and Porath, 1961).

The use of granulated agar gels for separating proteins was described by Polson (1961). He found that optimal conditions were obtained with gels containing 7 per cent agar, and then even a protein with a molecular weight of 490.000 could be separated from larger species. It was concluded that the separation was largely dependent upon differences in diffusion coefficients and to a lesser extent on differences in molecular weight. Lea and Sehon (1962) reported the fractionation of plasma proteins into two fractions with a gel made by copolymerization of acrylamide and methylene bis-acrylamide. Experiments were also made in gels synthesized by copolymerization of methylene bis-acrylamide with vinylethyl carbitol and with vinyl pyrrolidone. Acrylamide gels were also used to separate a protein mixture containing phycocyanin, phycoerythrin, hemoglobin, and cytochrome C (Hjertén and Mosbach, 1962).

The ability of gels to discriminate between solutes differing in size is not unique for water-swollen systems. Thus cross-linked polystyrene beads swollen in benzene were used for the fractionation of polystyrene in benzene solution (Vaughan, 1960). Cortis-Jones (1961) studied gel filtration in beds of cross-linked polystyrene swollen by different solvents and observed that both molecular sieve effects and sorptive forces influenced the elution behaviour of many solutes. In a column packed with granulated vulcanized rubber latex swollen in toluene a linear relationship was observed between the corrected elution volumes and the molecular weights of the solutes (Brewer, 1961).

[1] Manufactured by AB Pharmacia, Uppsala, Sweden.

CHAPTER 2

The preparation of dextran gels

According to Hermans (1949) the term gel should be assigned to systems defined by the following three criteria: a) they are coherent colloid disperse systems of at least two components, b) they exhibit mechanical properties characteristic of the solid state, and c) both the dispersed component and the dispersion medium should extend evenly throughout the system. This definition includes a very large number of systems of gels formed by crystallizable low molecular weight substances such as vanadium pentoxide or azometin, to macromolecular gels of the type formed by cooling solutions of gelatin or agar.

Only gels having good mechanical and chemical stability and which can be made in variants differing widely in content of dry substance are of interest here. They should in addition be xerogels, which means that the dispersion medium can be removed reversibly. These conditions are fulfilled by certain macromolecular gels especially those having covalent bonds throughout their three-dimensional networks. Gels of this type have been the subject of intensive studies since the middle of the nineteen thirties when their use as ion exchangers was discovered.

In making ion exchange resins two processes are most widely used, the condensation of phenols with formaldehyde and the copolymerization of styrene and divinylbenzene. The ion exchanging groups can be introduced either by using monomers containing the ionizing group or, in the case of cross-linked polystyrene, by substituting in the polymer itself (Helfferich, 1959). In the absence of ionized groups these types of polymeric networks do not swell in water.

For use as a molecular sieve in the gel filtration method a swollen network devoid of ionic groups gives the best results. For aqueous systems it is therefore necessary to use polymers containing hydrophilic groups of non-ionic character. Relatively few cross-linked polymers of this type have been described in the literature. Recently, Lea and Sehon (1962) have described the copolymerization to particulate gels of methylene bis-acrylamide with acrylamide, vinylethyl-carbitol and vinylpyrrolidone, respectively. A mixture of the first monomer and the crosslinking agent is available commercially and is used widely to produce gels for electrophoresis. Its use in a particulate form for separations is described by Hjertén and Mosbach (1962). The soluble polymers polyvinyl-pyrrolidone and polyvinylalcohol have also been cross-linked by heat-treatment and ionizing radiation.

The abundance of hydroxyl groups in polysaccharides imparts a high affinity for water. It has long been known that these polymers and also polyvinyl-alcohol can be cross-linked with aldehydes, particularly formaldehyde (Walker, 1953) in acid media. Numerous examples can be found in the patent literature. Cross-linking of cellulose and starch and their derivatives with epichlorhydrin or diepoxides has also been described in many patents. During the last few years this reaction has aroused much interest judging from the many publications which have appeared (e.g. Jones and Rayburn, 1961). The work of Deuel (1947) is also of interest. He cross-linked several polysaccharides with epichlorhydrin in alkaline media. Pectin, for example, was cross-linked to form a weak cation exchanger. An uncharged polysaccharide, a galacto-mannan from locust bean gums was also cross-linked (Deuel and Neukom, 1954). Dextran and dextran derivatives were similarly cross-linked in alkaline solutions (Ingelman 1946, personal communication). The synthesis of similar gels suitable for gel filtration and ion exchange has been described in patent specifications (Flodin and Ingelman, 1959; Flodin and Porath, 1961 a). The preparative methods will be described in more detail below.

The reaction between dextran and epichlorohydrin.

Dextran. The polysaccharide dextran used in this work was synthesized microbiologically by the action of Leuconostoc mesenteroides strain NRRL B-512 on sucrose. The product, the so-called "native dextran" is a very high molecular weight glucan containing about 90—95 per cent alpha-1,6-glucosidic bonds. The remaining bonds are mainly of a 1,3-glucosidic type. The native dextran is very heterogeneous and molecular weights ranging from 10-300 $\times 10^5$ have been found in fractions from the same parent sample (Arond and Frank, 1954).

The molecular weight of dextran can be reduced by partial hydrolysis of the glucosidic bonds at elevated temperatures in the presence of mineral acids (Grönwall and Ingelman, 1945). With suitable conditions almost any average molecular weight can be obtained.Complete hydrolysis yields only glucose. Hydrolysis does not reduce the heterogeneity sufficiently for many purposes but it can be made more homogeneous by fractional precipitation with ethanol (Ingelman and Halling, 1949). This treatment also serves another purpose, namely, to remove impurities present in the native dextran or formed during the hydrolysis. The final products are of a purity difficult to achieve with other polymers.

Of decisive influence on the properties of dextran are its molecular weight and molecular weight distribution. Three methods are used for this characterization, namely determination of the limiting viscosity number ($/\eta/$), the number ($\overline{M}_n$),

and weight ($\overline{M}_w$), average molecular weights. The ratio $\overline{M}_w/\overline{M}_n$ is a measure of the molecular weight distribution. For the synthesis of gels of high water regain (for definition see page 31), high molecular weight dextrans are the most suitable. To obtain high yields of insoluble gel the heterogeneity should be as low as possible. The handling of concentrated dextran solutions is facilitated if their viscosity is not high; this however, necessitates the use of low molecular weight dextrans. Thus, for the synthesis of gels with water regain values between 2 and 5, a weight average molecular weight of 30-50,000 is suitable while fractionated native dextran is suitable for gels with a water regain value of 20. For the former gel types a ratio $\overline{M}_w/\overline{M}_n$ of 2-3 has been found satisfactory.

The reactivity of dextran. The reducing end-groups, of which there is only one per molecule, are highly reactive. They are, however, relatively so few in high molecular weight dextrans as to be of little importance in the cross-linking reaction, except as sites for undesirable side-reactions as discussed below. The reactivity of dextran is therefore largely determined by the hydroxyl groups, of which there are three per glucose residue except at the branching points and in the terminal glucose residues. Many reactions characteristic for alcohols could be utilised but for gel formation epoxide addition with the formation of ethers has been found most suitable for a number of reasons. Ethers are relatively unreactive and thus give stable derivatives. The reaction can be performed in aqueous solutions under mild conditions.

An alcohol and an α, β-epoxide react in the presence of hydroxyl ions according to the following reaction

$$(1)\quad \text{R-OH} + \underset{\diagdown\text{O}\diagup}{\text{CH}_2\text{—CH-R}_1} \xrightarrow{\text{OH}^-} \text{R-O-CH}_2\text{-CH(OH)-R}_1$$

With acid catalysts this product is also formed but a considerable part of the reaction takes place at the β-carbon atom. To unite two alcohols a bifunctional epoxide is required. Though epichlorohydrin is not a diepoxide it reacts as though it were. Its reactions with an alcohol in the presence of sodium hydroxide are given below:

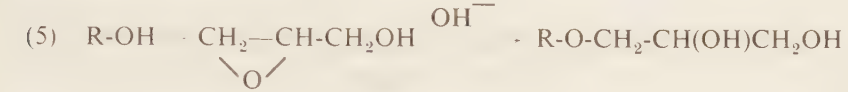
$$(2)\quad \text{R-OH} + \underset{\diagdown\text{O}\diagup}{\text{CH}_2\text{—CH-CH}_2\text{Cl}} \xrightarrow{\text{OH}^-} \text{R-O-CH}_2\text{-CH(OH)-CH}_2\text{Cl}$$

$$(3)\quad \text{R-O-CH}_2\text{-CH(OH)-CH}_2\text{Cl} + \text{NaOH} \rightarrow \text{R-O-CH}_2\text{-}\underset{\diagdown\text{O}\diagup}{\text{CH—CH}_2} + \text{NaCl} + \text{H}_2\text{O}$$

$$(4)\quad \text{R-O-CH}_2\text{-}\underset{\diagdown\text{O}\diagup}{\text{CH—CH}_2} + \text{HO-R}_1 \rightarrow \text{R-O-CH}_2\text{-CH(OH)-CH}_2\text{-O-R}_1$$

Two alcohol molecules are thus connected through a glyceryl bridge. When the alcohol is polyfunctional with respect to its hydroxyl functions, such as in

the case of dextran, a molecule can be connected to other molecules at many sites. Under such conditions a three-dimensional network will be formed since theoretically, the basic requirement for the initial formation of a network from large molecules is one bridge between two primary molecules (Flory, 1953). It was mentioned above that one advantage with the epoxide reaction is that it can occur in aqueous solutions. This condition depends on the very polar nature of dextran, requiring strongly polar solvents. Water also reacts with epoxides giving side-reactions of which the most important is the hydrolysis to glycerin-α-monochlorohydrin. Under the action of sodium hydroxide it forms glycidol, which in turn can be hydrozed to glycerin or joined to the hydroxyls of dextran according to the reaction:



(5) $R\text{-}OH + CH_2\text{—}CH\text{-}CH_2OH \xrightarrow{OH^-} R\text{-}O\text{-}CH_2\text{-}CH(OH)CH_2OH$

The same product is also obtained by hydrolysis of the dextran-bound epoxide shown in reaction 3. The situation becomes still more complicated when it is realised that the hydroxyl groups of the glycerol residues and glyceryl bridges can participate in further reactions. Due to the relatively low substitution obtained (see page 28) it is probable that reactions 2-5 are the dominant substitution reactions.

Reaction conditions. The technique used for cross-linking dextran was similar in all experiments reported here and the detailed description of one experiment together with the effect of varying the conditions will therefore suffice for a satisfactory picture of the synthetic method.

A reaction flask was fitted with a thermometer and a stirrer of anchor-type driven by a strong motor. 500 grams of a crystalline dextran with limiting viscosity number 0.19 corresponding to a weight average molecular weight of 40,000 were wetted with 200 ml water. In this way clump formation was avoided when 300 ml 5 molar sodium hydroxide were added. When solution was complete 135 grams of epichlorohydrin were added and stirred into the viscous dextran solution. The reaction flask was then put in a water bath at 40°C and the mixture stirred until it solidified into a solid mass or block of gel (hence the term "Block polymerization") which occurred after one hour. The reaction was allowed to continue for 24 hours and then the temperature of the bath was raised to 70 C. After standing at this temperature overnight the lid was removed and the brittle gel disintegrated and transferred in portions to a Waring blendor. After grinding to small particles under water it was neutralized with hydrochloric acid and sedimented three times in an excess of water to remove fines. The soluble reaction products were then washed out on a filter. On the filter the gel particles were gradually dehydrated with portions of ethanol and finally they were dried overnight in a vacuum oven at 70 C. The yield was 576 grams of a product with a water regain of 2.5 grams per gram dry substance.

The influence of varying dextran concentrations and amounts of epichlorohydrin on the water regain is illustrated in Fig. 1. It can be seen that at high

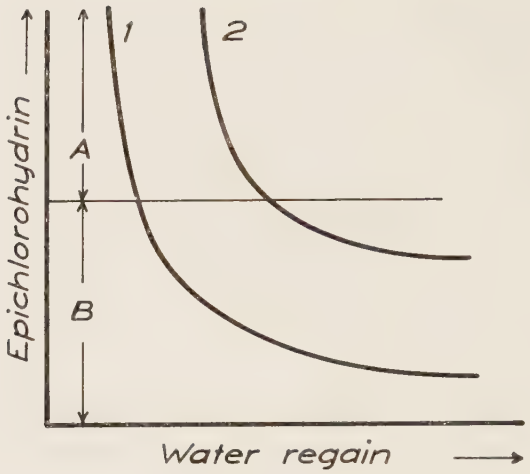


Fig. 1

A schematic representation of the variation in the water regain value of dextran gels with the epichlorohydrin concentration. Curves A and 2 illustrate the behaviour with a high and a low dextran concentration, respectively. In region A the water regain value is relatively independent of the amount of epichlorohydrin; in region B the concentration of the reactants have a profound influence on the swelling of the gel product.

epichlorohydrin concentration levels the water regain is insensitive to the amount of epichlorohydrin. On the other hand in region B even small changes either in dextran or in epichlorohydrin concentration, cause dramatic changes in water regain. The yield of gel product decreases with increasing water regain towards nil in the limiting case of a completely soluble polymer. For optimal reproducibility it is best to work in region A where only adjustments of the dextran concentration are necessary to obtain a particular water regain value.

Table 1 shows the variation of the water regain with varying dextran con-

Table 1.

The variation of water regain with dextran concentration.

Molecular weight of dextran: $\overline{M}_w = 40{,}000$.
Weight of epichlorohydrin relative to the weight of dextran: 19 %

Dextran conc.	Water regain
%	g/g
56	1.8
32	4.1
28	4.9
20	14

Table 2.

The variation of water regain with amount of epichlorohydrin.

Molecular weight of dextran: $\overline{M}_w = 40{,}000$.
Initial dextran concentration = 56 % (w/w)
Epichlorohydrin amounts are given as per cent of dextran.

Epichlorohydrin	Water regain
%	g/g
35.5	2.1
30	2.6
18	3.0
6	10

Table 3.

The variation of water regain with the molecular weight of dextran.

Initial dextran concentration = 20 % (w/w).

Molecular weight $\overline{M}_w$	Epichlorohydrin % of dextran	Water regain g/g
$1.8 \cdot 10^6$	16	8
$4 \cdot 10^5$	20	7.5
$4 \cdot 10^4$	20	14

centration for a dextran with molecular weight 40,000. Table 2 gives the variation with epichlorohydrin concentration. In all cases the amount of sodium hydroxide was calculated to give an excess of about 10 per cent. The influence of the molecular weight of a parent dextran on the capacity of a gel to imbibe water is illustrated in table 3. At low dextran and epichlorohydrin concentrations no gel was obtained, though polymerization of the dextran is known to have occurred. With other parameters constant, reduction of the molecular weight of the dextran leads to a higher water regain. A high water regain value results in an increase in the amount of dextran not incorporated in the network.

The reaction of epichlorohydrin and dextrans is exothermic, and large amounts of heat must be removed by conduction. This becomes a problem with high dextran concentrations and large amounts of epichlorohydrin, since after gel formation heat conduction from the inner parts of the reaction vessel is poor. This may lead to an uncontrollable reaction in the interior resulting in unsatisfactory products. By starting off the reaction at a low rate, that is at a low temperature, this effect can be minimized. When most epichlorohydrin has reacted the temperature is raised to complete the reaction in a reasonable time. Experience has shown that the optimal temperature for the first stage is between

20—40°C and for the second stage, the "hardening", about 70°C. Too high temperatures cause oxidation and discoloration of the product.

As mentioned above the glucose residues at the reducing ends of the dextran molecules are reactive. Under the conditions for the cross-linking reaction they are liable to be destroyed and it has often been found that the lower the molecular weight of the dextran, the stronger the discoloration. Furthermore, it is known that polysaccharides are oxidized by oxygen in alkaline media. Attacks may thus occur anywhere along the chains. Complicated reactions take place and the product becomes discoloured and contains increased amounts of carboxyl groups. These two side-reactions lead to inferior products, but can be minimized by the presence of sodium borohydride, which reduces the end-groups and prevents oxidative attack. The borohydride is preferably dissolved in the sodium hydroxide solution before dissolving the dextran. An amount corresponding to 0.2—0.5 per cent of the weight of dextran usually suffices to give a white product with few carboxyl groups. Excess borohydride results in hydrogen evolution and foam formation during the reaction.

The reaction between other polysaccharides and epichlorohydrin.

Under conditions analogous to those for dextran other polysaccharides can be cross-linked with epichlorohydrin. Starch can be used to produce gels of high water regain, while dextrins are more suitable for low water regain types because their viscosity is much lower in concentrated solutions. Starch is more difficult to purify than dextran and the gels prepared from washed starches and dextrins were found to have inferior properties, as regards the presence of ionized groups.

Starch is easily oxidized in alkaline media and the presence of sodium borohydride was essential to obtain usable gels. Some reacting conditions and water regain values are given in table 4.

Table 4.

Water regain values for starch and dextrin gels.

Polysaccharide		Epichloro-hydrin	NaOH		Water regain
Type	g	g	Molarity	ml	g/g
Starch	600	300	1.4	2500	5.1
Dextrin	100	25	4	150	1.7
„	100	40	5	100	1.2

Some experiments were made with alkalicellulose and alkalidextran (dextran forms an insoluble alkali complex in sodium hydroxide solutions of concentrations higher than 40–45 per cent). As was mentioned in the introduction very efficient insolubilization is obtained with epichlorohydrin. The products, however, were inferior to solution polymerized gels as regards gel filtration properties. A plausible explanation is that only the amorphous regions are accessible to the reactants. Thus, after cross-linking, some parts of the particles are still crystalline and not accessible by diffusion to water or solutes present in the surrounding medium.

The reaction with epichlorohydrin can also be performed with soluble derivatives of polysaccharides. The hydroxyethyl and the alkyl hydroxyethyl ethers of cellulose were cross-linked in this way. Since these derivatives have very high viscosities in solution, only gels with high water regain values were obtained. Another ether of interest, because it gives gels swelling in both water and ethanol, is the β-hydroxypropyl ether of dextran. Wide variations in water regain are possible with this ether, since the dextran to be substituted can be chosen at will.

The reaction between polyhydric alcohols and epichlorohydrin.

A necessary condition for the formation of three-dimensional networks is that at least one of the reactants has a functionality higher than two and the other is at least bifunctional (Flory, 1953). Thus glycerin and the higher polyhydric alcohols should be capable of forming networks. In this laboratory sucrose and sorbitol have been polymerized with epichlorohydrin with resulting gels. The sorbitol gels are of special interest since they contain only -C-C- and -C-O-C- and no glycosidic bonds in the network and should thus be more stable than polysaccharide gels in the presence of acids.

The low molecular weight of the reactants require, however, reaction conditions other than those reported above and the side reactions also become more pronounced.

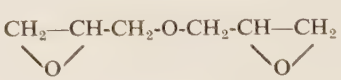
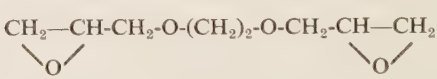
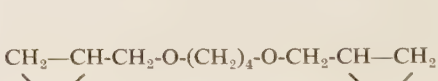
Polycondensation of sorbitol and epichlorohydrin. To 200 grams of a 70 per cent solution of sorbitol in water 150 grams 50 per cent sodium hydroxide solution were added. While stirring at room temperature 160 grams epichlorohydrin were added slowly. After stirring the mixture overnight it was heated to 60 C for 24 hours. The gel formed was disintegrated, washed and dehydrated with acetone. It had a water regain of 8 grams per gram of dry substance.

Reactions with diepoxides.

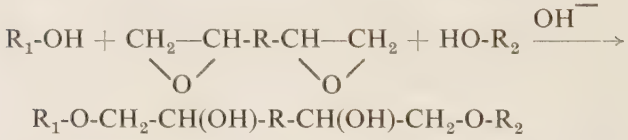
The reaction of polysaccharides with glycerin 1,3-dichlorohydrin is similar to that with epichlorohydrin since in the first step HCl is split off with resulting formation of epichlorohydrin. Many of the diepoxides can also be used, pro-

Table 5.

Diepoxides

Name	Formula	Synthetic method ref.
1,2 : 3,4-diepoxybutane (DB)		Prevost (1928)
Bis-epoxypropyl ether (BE)		Castan (1938)
Ethylene glycol bis--epoxypropyl ether (EBE)		Cohen and Haas (1953)
1,4-Butanediol bis--epoxypropyl ether (BBE)		Cohen and Haas (1953)

vided they are sufficiently soluble in the reaction mixture. This condition and the requirement of a non-electrolyte character make some aliphatic α, β-diepoxides particularly useful. In table 5 their formulas and methods of synthesis are given. They react with alcohols in alkaline media as follows:



Only catalytic amounts of hydroxyl ions are necessary for the reaction between polysaccharides and the diepoxides. In other respects the procedure is similar to that for dextran and epichlorohydrin. In table 6 some reaction conditions and resulting water regain values are given. In accordance with the finding by Croon and Lindberg (1956) that the primary hydroxyl groups in cellulose react about five times faster with epoxides than do the secondary hydroxyl groups, it was found that starch and hydroxyethyl cellulose gave gels at lower temperatures than dextran.

A polymer with a high content of hydroxyl groups, polyvinylalcohol, did not give satisfactory gels with epichlorohydrin due to its insolubility in sodium hydroxide solutions. With diepoxides porous gels, which were remarkable because of their elasticity, were obtained. The latter property made them extremely difficult to disintegrate into particles of size suitable for gel filtration.

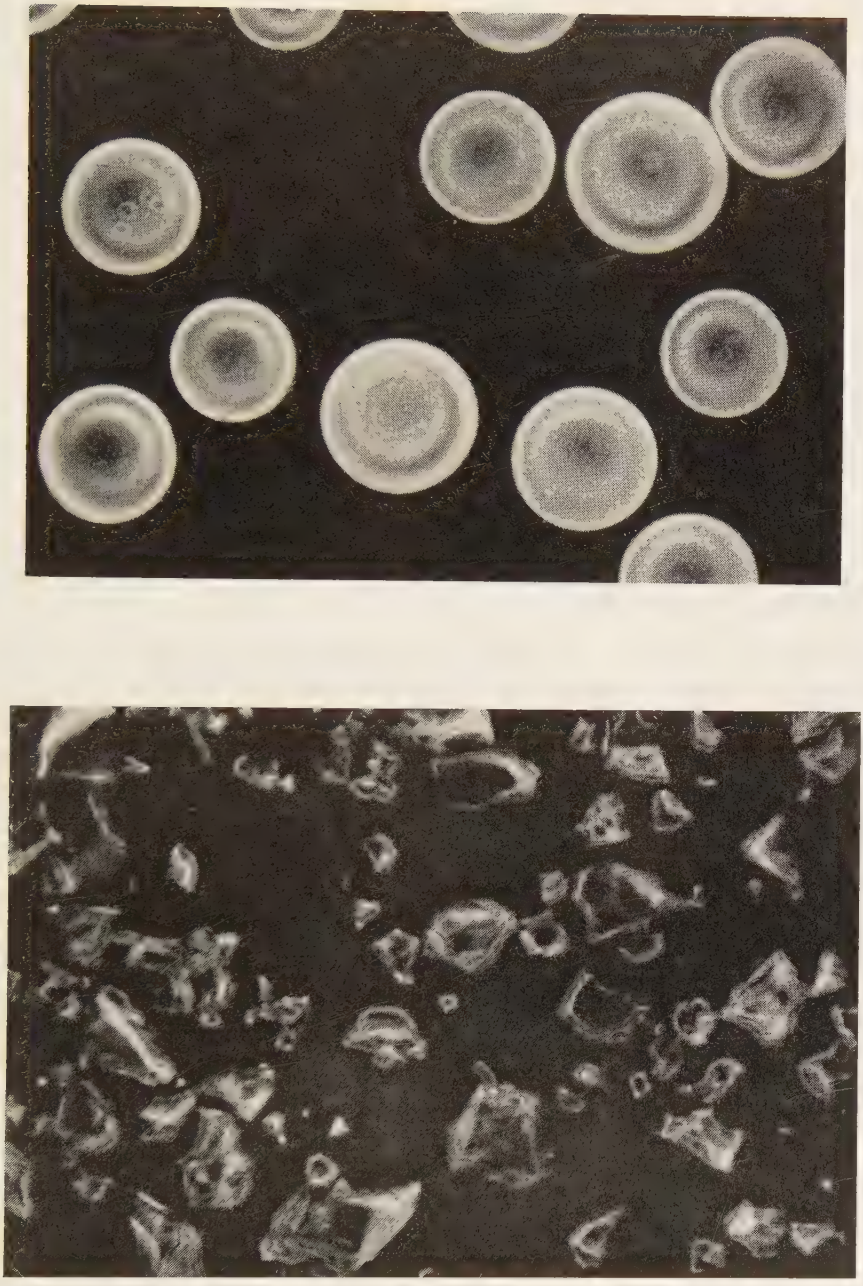


Fig. 2

Dark ground photomicrographs of swollen dextran gel particles. The block polymerizate Sephadex G-25, Fine (above) and the bead polymerized Sephadex G-100, 200—270 mesh (below). 1 cm corresponds to 100 μ.

On the other hand, by performing the polymerization in thin layers, membranes of excellent strength could be made. This is in contrast to similar polysaccharide membranes which were brittle and therefore difficult to handle.

Table 6.

Reactions between dextran and diepoxides.

For abbreviations see table 5.

Dextran		Diepoxide		NaOH		Water regain
$\overline{M}_w$	g	Type	g	Molarity	ml	g/g
$4 \cdot 10^4$	25	DB	7.3	4	25	1.6
$4 \cdot 10^4$	54	BE	15	2	53	2.7
$4 \cdot 10^4$	50	EBE	50	4	50	1.5
$4 \cdot 10^4$	200	BBE	100	4	200	2.1

Polymerization in bead form.

When polymerization is done by the method described on page 17 the polymer forms as a solid mass or block which on disintegration yields particles of irregular shape as shown in Fig. 2. When these are packed in columns the resistance to flow through the bed is rather high, particularly with gels of high porosity, i.e. those of high water regain. The particles are elastic and thus easily deformed with a high pressure drop along the column, and eventually complete clogging of the column may occur. By dispersing the reaction mixture into drops in an inert dispersion medium the reaction can take place in these and spherical gel particles are formed. This is illustrated in Fig. 2.

The spherical particles or beads offer considerably lower resistance in a packed bed and consequently much more porous gels can be used (see chapter 8). Bead polymerization also offers other advantages. It was mentioned above that dissipation of heat was one of the problems in block polymerization. With small gel particles suspended in an inert dispersion medium, e.g. a hydrocarbon, all heat evolved is easily transferred to the walls of the reaction vessel. Another advantage is the possibility of producing directly a polymerized particle of a size useful for chromatography. The troublesome grinding and sieving processes necessary for block produced gels are thereby obviated.

Derivatives of dextran gels.

The number of hydroxyl groups is not changed appreciably by the cross-linking reactions. Their high proportion is responsible for the affinity for

water and they are also reactive sites where further substitutions can be effected. Their reactivity is approximately equivalent to that of the hydroxyls in the parent polysaccharides. Thus, esters and ethers can be formed easily.

To obtain even substitution the reaction is preferably carried out in such a manner that the network remains swollen throughout the reaction. Since relatively few solvents swell dextran gels (see chapter 3) the choice is limited to water, formamide, dimethylsulfoxide and a few less important ones. To minimize any side reactions it is liable to produce, the amount of solvent should be as small as possible. Such reactions are conveniently carried out with the gel in a semi-dry condition, that is the amount of solvent used is less than can be imbibed by the gel.

It is also important to carry out the substitution reactions under conditions which do not favour break-down of the network. Thus, strongly acidic conditions and high temperatures should be avoided. Etherification in strong alkali at temperatures over about 90 C has also been found to give inferior products. In general, the reactants should be chosen so that the reactions can be carried out at room or only slightly elevated temperatures.

Esters. With formamide as the swelling agent esterification can easily be performed with acid chlorides or anhydrides. Pyridine is a suitable proton acceptor and for the acetylation of dextran gels a procedure similar to that described by Jeanes and Wilham (1952) for dextran can be used. Highly substituted esters are easily obtained and they have swelling properties quite different from that of the original gel. For example they do not swell in water but in non-polar solvents such as chloroform and toluene.

Ethers. Of considerable importance are ethers containing ionized groups which give the gel ion exchange properties. Thus, ethers containing carboxyl, sulfonic and diethylamino groups have been synthesized from dextran gels and chloroacetic acid, 2-bromoethane sulfonic acid, and diethylaminoethylchloride, respectively. The reactions were carried out in the presence of alkali. Due to hydrolysis occuring as a side reaction the amounts of water had to be kept low in order to obtain high degrees of substitution. It was found advantageous to perform the substitution with the swollen gel particles dispersed in an inert solvent such as a hydrocarbon. Effective temperature control is obtainable in this fashion and the products become more evenly substituted.

The carboxymethyl ether. To a flask containing 400 ml benzene were added 100 grams of a dextran gel of water regain 2.5 and then 180 grams of a 50 per cent sodium hydroxide solution. The flask was placed in a water bath at 50°C and 112 grams of chloroacetic acid were added in portions while stirring. The additions were made sufficiently slowly to prevent the temperature from exceeding 55°C. After five hours the product was isolated. It had an ion exchange capacity of 3.0 milliequivalents per gram dry substance.

The introduction of ionized groups into dextran gels causes dramatic changes in the swelling behaviour. In contrast to the unsubstituted gels the degree of swelling depends on the ions present in the surrounding solution. Thus, in distilled water the monovalent salts imbibe more than twice as much water as the original gel. With increasing ionic strength they shrink and also in other respects behave in a manner analogous to ion exchange resins (Helfferich, 1959). The reason for the greater swelling in distilled water as compared with the unsubstituted gels is that the macromolecular chains in the latter are not completely expanded but become so under the influence of electrostatic repulsions between neighbouring charged groups.

Structures and properties

Structure.

The most probable chemical structure of a small part of the network formed on cross-linking dextran with epichlorohydrin is shown in Fig. 3. It was deduced from the known reactions of epichlorohydrin with alcohols shown on page 16. The two most probable reaction products are illustrated, namely the cross-linking proper and the formation of glyceryl side-chains. A more detailed study of the reaction products is difficult. The elementary composition and also the number of hydroxyl groups change little during the reaction. It should be possible, however, to study the products formed after total acid hydrolysis of the glucosidic bonds under conditions not affecting the ether linkages. A few preliminary experiments in this direction have been made under the conditions

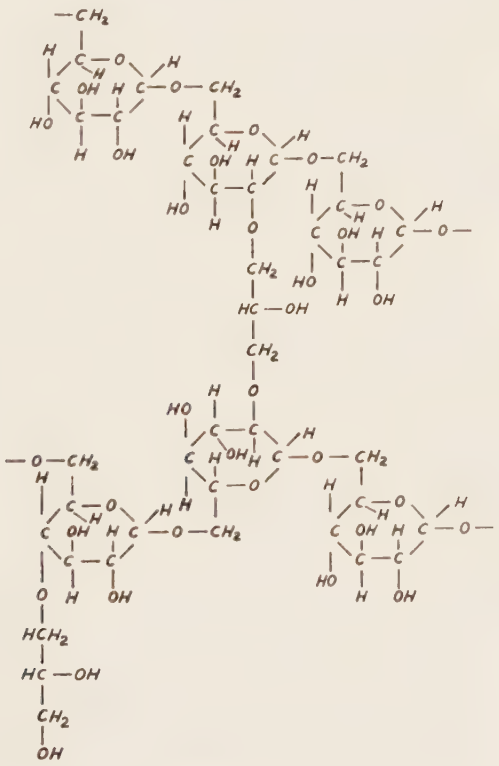


Fig. 3

Structural formula showing the essential features of a dextran gel cross-linked with epichlorohydrin.

described for analytical total hydrolysis of dextran by Dimler et al (1954). Paper chromatography of the hydrolysates gave in all cases very strong glucose spots and at least three minor spots with lower R_f-values. These were not identified but one of these spots may represent two glucose residues joined by the cross-linking bridge. Thus it might be possible to determine the number of cross linkages.

The number of non-substituted glucose residues can be determined by periodate oxidation. This is a technique widely used for the determination of the degree of branching of dextrans (Jeanes and Wilham, 1950). Glucose residues in a 1,6-glucan consume two moles of periodate with the formation of formic acid. When the hydroxyls in positions 2, 3 or 4 are substituted no formic acid is formed. The results of periodate oxidation of some commercially available types of dextran gels are shown in table 7. They are in agreement with paper chromatographic evidence that a considerable part of the glucose residues was unsubstituted.

Table 7.

Results of periodate oxidation of commercial types of dextran gels compared with values for water regain and wet density.

Sephadex type	Water regain g/g	Wet density g/ml	Moles IO_4^- per mole glucose	Moles HCOOH per mole glucose
G-25	2.5	1.12	1.05*	0.393*
G-50	5.0	1.06	1 36*	0 580*
G-75	7.5	1.03	1.33	0.574
G-100	10.0	—	1.55	0.717
G-200	20.2	—	1.72	0.811

* Corrected for IO_4^- consumption by the end groups.

Dextran molecules in solution are randomly coiled. Under the conditions prevailing in the cross-linking reaction the molecules are heavily entangled and it is thus not meaningful to consider individual molecules. A more useful concept is the glucose residue density which is constant throughout the solution when the reaction starts. In the cross-linking reaction, thermal agitation causes glucose residues from two chains to move into positions where they can react. As the reaction proceeds more and more pairs of glucose residues from different dextran chains become fixed relative to each other. Due to the entangling of the chains it seems reasonable to assume that loops etc., which may be called "pseudo-cross-linkages" are formed as a consequence of fixation of more points in the chains. Both the true and the "pseudo" cross-linkages will restrict the movement of the chains. When the final gel product is exposed to a solvent the glucose residues strive to separate as far as possible as a consequence of sol-

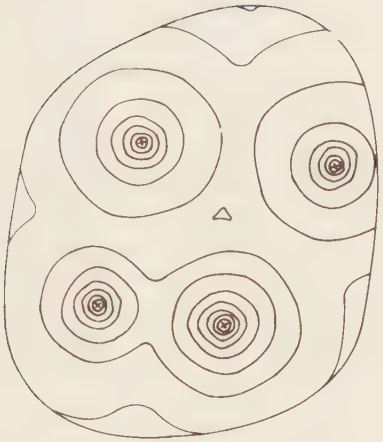


Fig. 4

Hypothetical two-dimensional projection of the microheterogeneous structure of a gel particle. The crosses indicate cross-linking sites. The lines represent contours of equal glucose residue densities.

vation. At the cross-linkages they are fixed but in other regions movement is possible. The better the solvent, the greater will be the separation, and consequently the chains will become more stretched. Thus, it seems reasonable to assume that in the swollen state there are within the gel microheterogeneities which can be expressed as gradients of glucose residue density from the cross-linking sites outwards. The lowest density will then be somewhere midway between two sites. A schematic representation is shown in Fig. 4.

A consequence of this view is that with the same cross-linking agent the smallest distance between two chains is the same irrespective of the degree of cross-linking. The number of cross-linkages, however, has a pronounced effect on the minimum glucose residue density. The lower the number the greater the volume of solvent imbibed and hence the steeper the glucose residue density gradient.

Chemical properties.

The reactivity of the dextran gels is similar to that of dextran. The most easily attacked points are the glucosidic bonds and the hydroxyl groups. The former, as mentioned previously, hydrolyze in the presence of strong acids at elevated temperatures. At room temperature the stability of the gels is good even under highly acidic conditions. Thus, a gel did not change its properties after storage for half a year in 0.02 molar hydrochloric acid at room temperature (Cruft, 1961).

The hydroxyl groups can undergo many of the usual reactions such as esterification or etherification (see page 25). The adjacent position of the

hydroxyls in the glucose residues renders them susceptible to periodate oxidation as described on page 28.

Heating the xerogel or the swollen gel to temperatures higher than 120°C causes break-down in an unknown manner. The color of the product and the chemical similarity to sugars suggests caramelization, dehydration and/or oxidation. The reaction in alkaline media also causes similar side reactions. It is known that polysaccharides are susceptible to oxidation by oxygen in the presence of alkali. That this is the case in the cross-linking reaction is evident from the formation of carboxyl groups and the yellow-brown color of the product. When performed in the presence of sodium borohydride, however, the formation of these is largely suppressed. In addition the borohydride reduces the end groups, the reactivity of which is probably responsible for most side reactions.

The dextran gels are stable towards mild oxidizing and reducing agents.

Physical properties.

In the dry state dextran gels are hard and tough powders with a density of 1.64—1.65. In the swollen state, however, they are brittle and disintegrate easily when sqeezed. Depending upon the water regain they are more or less elastic. As mentioned above (page 24) the elasticity of gels with very high water regain values is so large as to prohibit their use in chromatographic columns, since their compressibility leads to inconveniently low flow rates. The bead polymerizates are better in this respect and the bead polymerized Sephadex G-200 gives even better flow rates than ground block polymerized Sephadex G-75 of corresponding particle size.

Swelling.

The swelling of dextran gels in contact with solvents depends both upon the degree of cross-linking and the solvation power which is a function of the solvent-gel interaction. Since the gels are polar they only swell in the presence of polar solvents such as water, dimethylsulfoxid, formamide, and glycol. Less polar water miscible solvents such as ethanol and acetone effectively dehydrate the water swollen gels. Swelling and desolvation of gels are the same phenomena as dissolution and precipitation of soluble polymers. Solvation begins at the outermost "layer" of the particles, which then expands exposing the next layer to solvent and so on until completion of the process. The rate of swelling is governed by the solvation power and the diffusion rate of the solvent molecules.

The amount of solvent imbibed by a gel is an important measure determined by the number of cross-linkages and the glucose residue density gradients. It is a useful indicator of the relative porosity of gels synthesized in the same

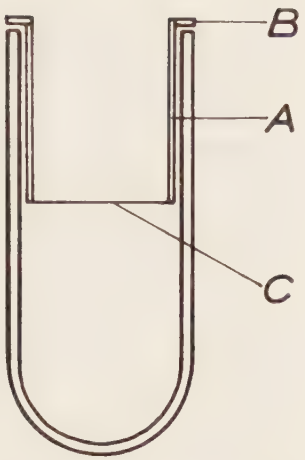


Fig. 5.

Arrangement for water regain determination. A plastic adaptor (A) with a flange (B) and a 400 mesh nylon net (C) is inserted into the centrifuge tube. During centrifugation a rubber cover is applied.

manner, i.e. with the same basic structure. For dextrans gels the imbibed water is expressed as the water regain value (in grams per gram dry gel) of the gel and can be conveniently determined by a procedure modified after that of Pepper et al. (1952).

Weter regain determination. The gel is allowed to swell in water for 24 hours. About 10 ml is then transferred into an weighed adapter (see Fig. 5) which is placed in a centrifuge tube. The liquid in the void space is centrifuged down through the filter at 1000—2000 rpm for 20 minutes (radius 15 cm). The adaptor with its contents is then weighed and the contents transferred to a beaker and dried to constant weight at 105°C. The water regain is expressed as grams of water imbibed per gram dry gel (Granath and Flodin, 1961).

The wet density is determined by weighing centrifuged gel into a pycnometer, which is then filled with water, com pletely deaereated and weighed.

Water regain values have been given in the chapter dealing with synthesis. For the commercially available types of dextran gels (Sephadex), water regain and wet density values are given in table 7 on page 28.

An approximate measure of the swelling is obtained by suspending a weighed amount of dry gel in water in a graduated cylinder and reading the volume of the sediment after swelling equilibrium has been reached. The packed volume may vary between different determinations or between gels of different particle size and density. The values obtained, however, are quite useful since they yield an approximate figure for the volume a certain weight of gel will occupy in a packed column. Some values are given in table 10 on page 42.

Solubility.

The solubility of the dextran gels have been determined at two temperatures. At room temperature samples were stirred in an excess of distilled water.

Every 24 hours the gel was washed and resuspended in fresh water. The pooled extracts were evaporated and the carbohydrate content determined by the anthrone reaction (Scott and Melvin, 1953). It was found that 0.1—1 per cent of the gel had been extracted during the first 24 hours. The amount varied with the gel type and batch. After 4—5 days constant values were obtained, the release of soluble carbohydrate material being 0.002—0.003 per cent per day. Values within this range were obtained for the three types of dextran gel studied, namely Sephadex G-25, G-50 and G-75.

At 65°C higher values were obtained after extraction in a Soxhlet apparatus. The 24 hour extracts were evaporated and the dry weight of soluble material determined. For Sephadex G-25 a constant release of soluble material was obtained after 4 days amounting to 0.01 per cent per day.

Theory of molecular sieve action.

It is a well-known fact that ions and small molecules can diffuse in the water imbibed by a gel. In a study on polyacrylamide gels it was established that all water was equally accessible to all the low molecular weight solutes studied (White and Dorion, 1961). On the other hand it has often been found that large molecules do not enter gels presumably due to the steric hindrance of the gel network. In ion exchangers of the resin type such exclusion effects have been observed repeatedly (Helfferich, 1959). The problem remains of whether there is a sharp boundary or a gradual transition for molecules of intermediate size.

In a comparison between the space available to ions in dextran gels it was found that alkali metal chlorides occupied smaller volumes than did tritiated water. Still more interesting was the observation that there seemed to be a definite relation between the hydrated ionic radius and the water space available (Marsden, personal communication). Small molecules, such as sugars, apparently do not distribute in all the water in the gel. Thus, sucrose in equilibrium experiments changed its concentration in the outside water in a proportion indicating that only 70 per cent of the imbibed water was accessible.

In column experiments, tritiated water, ions, sugars and other molecules known to penetrate gels are eluted generally as symmetrical peaks. They show the dependence on elution rate and particle size to be expected from a diffusion controlled process. In other words, their elution profiles take the form of error curves the width of which depends on the deviations from local equilibrium. In column experiments it has been observed repeatedly that larger molecular species up to the size of serum albumin and gamma-globulins behave in the same way.

For penetrant species, large molecules are eluted faster than small ones and the greater the size difference, the greater the difference in elution rate. Dextrans

show a smooth transition towards higher migration rates with increasing molecular weight up to the limit of total exclusion (Granath and Flodin, 1961).

All these facts and observations taken together lead to the conclusion that for any particular gel a molecular species of a certain size (and probably form) can diffuse only into limited regions of the gel. The concept of glucose residue density gradients as outlined above can be used to explain this. As a molecule diffuses through the gel it encounters regions so densely populated with dextran chains that they block its passage. Around each cross-linking site there is a forbidden region which varies in size with the size of the molecular species. In all other parts of the gel solutes can diffuse more freely. In the limiting case of a solute too large to enter, the whole gel particle is a forbidden region.

In order to interpret the elution behaviour in columns it is necessary to postulate equilibrial or at least quasi-equilibrial conditions, and to assume a rapidly decreasing diffusion coefficient within regions of very small dimensions. In studies on the diffusion of large molecules in the presence of varying concentrations of hyaluronic acid, Laurent (personal communication) found an exponential relation between the diffusion coefficient and the polymer concentration. The same interrelation had earlier been found between the sedimentation coefficient and the concentration (Laurent and Pietruszkiewicz, 1961). Since a large number of cross-linking sites are present in dextran gels, the glucose residue density gradients must have on the average very small linear dimensions. In these regions the diffusion coefficients can be expected to decline rapidly towards limiting values. This concept of diffusion coefficient dependence upon the gradients is illustrated in Fig. 6. It is only when a molecular species closely

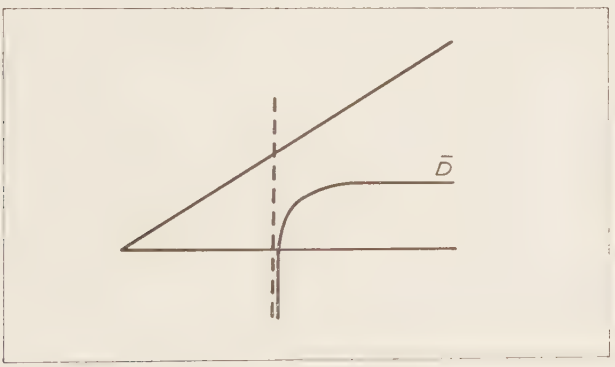


Fig. 6.

Diagrammatic representation of the hypothetical changes in the diffusion coefficient ($\bar{D}$) of a solute diffusing into a gel particle. The resistance to diffusion is relatively small in the wider region (right) but increases rapidly near the broken line, which indicates the limit of penetration for the solute.

approaches an unpenetrable region that its diffusion coefficient should decline significantly.

Thus, the diffusion of a solute into a gel particle may be supposed to occur in two stages. In the first stage restriction is virtually absent but the distances over which the solute has to diffuse are of the same order of magnitude as the gel particle itself. In the second stage the solute becomes more and more restricted until it can diffuse no further. This takes place over such short distances that the time for attainment of equilibrium is short. This reasoning will be clarified by considering the relation for the half-time of self-diffusion in spherical particles of radius r_o. This can be expressed as

$$t_{0.5} = 0.03 \frac{r_0^2}{\overline{D}}$$

where $\overline{D}$ is the diffusion coefficient in the gel (Helfferich, 1959). If it is assumed that the free diffusion coefficient is largely unchanged in the gel particle (first stage), serum albumin ($D \sim 10^{-6}$) would reach half-equilibrium in 3 seconds in particles with a radius of 0.01 cm. and in 0.03 seconds with a radius of 0.001 cm. (Swollen Sephadex particles have radii in the range 0.001—0.01 cm.) Thus, the distance over which a solute has to diffuse will have an enormous influence on the time for equilibration. It is also evident that a considerable decrease in diffusion coefficient does not greatly influence the equilibration time, if it only occurs over a very short distance. The conclusion to be drawn from the reasoning above is that the rate-determing stage is the diffusion over the relatively long distances, where little restriction occurs.

To summarize, a hypothesis is put forward to explain the molecular sieve action of dextran gels. It is supposed that microheterogeneities which are produced during synthesis greatly affect the diffusion of solutes inside the gel particles. The rate-determining stage is the diffusion in regions of relatively unrestricted diffusion. Each molecular species can only diffuse up to a "forbidden region" surrounding each cross-linking site. The magnitude of this forbidden region and hence minimum distance of approach depends on the steric properties of the solute itself.

Since the solute concentrations in the penetrable regions of the gel particles and in the surrounding solution are likely to be equal, the distribution coefficient, defined in chapter 4, indicates that fraction of the imbibed water which is penetrable for the solute. On the basis of a mechanism, similar to the one described above, Porath (1962) has derived an expression relating the distribution coefficient to the molecular weight of the solute and the water regain of the gel.

Theory of gel filtration

The ability of gels to distinguish between molecular species differing in size can be utilized to perform separations in a number of ways. By contacting the swollen gel with a solution in a counter-current system effective separations can be achieved. Such a procedure is, however, technically rather troublesome. By far the simplest and most efficient way to effect separations is to use a chromatographic technique with small gel particles packed into a column. A considerable number of publications have already appeared reporting various fractionations. These will be referred to in detail in later chapters.

The method differs in some respects from other chromatographic processes, such as partition or adsorption chromatography. It is noteworthy that the elution volume of all solutes is smaller than the bed volume. The gels are also remarkably insensitive to variations in solute concentration, which means that sorption isotherms are linear over wide concentration ranges. With many other systems they are linear only at low concentrations. These differences form the basis for the terminology. Thus, chromatographic procedures in which the molecular sieve effects of gels are utilized have been named gel filtration. Other synonyms are gel filtration chromatography (Strain, 1961) and molecular sieve chromatography (Fasold et al., 1961).

The ideal process.

It is usual in chromatography to define a parameter, the distribution coefficient (K_d), which is the ratio between the concentrations of a solute in the mobile and stationary phases. In gel filtration a similar parameter is very convenient. The stationary phase is then the water imbibed in the gel particles and the mobile phase the water moving in the void space between the particles. The physical concept of the distribution coefficient in terms of the molecular sieve hypothesis (page 32) is the volume fraction of the imbibed water, which is available to the solute.

In terms of the distribution coefficient (K_d) the elution volume of a solute is

$$V_e = V_o + K_d \cdot V_i$$

where V_o is the void volume of the column and V_i the volume of the water imbibed by the gel particles.

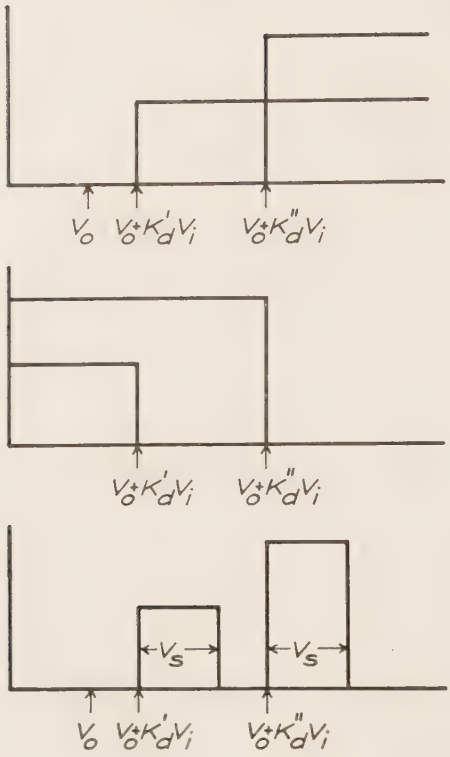


Fig. 7.

Schematic representation of the "ideal" behaviour of the solutes in a column. Upper diagram: frontal analysis. Middle diagram: frontal analysis interrupted and eluant added. Lower diagram: elution analysis of a sample of volume V_s. For further explanation see text.

If the density of water is takes as unity the imbibed volume is

$$V_i = a \cdot W_r$$

where a is the weight of dry gel and W_r is the water regain (page 31). Thus tha distribution coefficient becomes

$$K_d = \frac{V_e - V_o}{V_i} = \frac{V_e - V_o}{aW_r}$$

which can be obtained from experimental data. For determination of these values see page 47.

Since, as mentioned above, the sorption isotherms are linear over wide concentration ranges, the distribution coefficient remains constant under most conditions. This is a valuable asset as regards the practical applications of the process. Under ideal conditions, that is in the absence of zone spreading, frontal analysis of a solution containing two solutes with distribution coefficients K_d' and K_d'' yields an elution diagram as shown in Fig. 7. The two solutes appear in the effluent at the volumes $V_n + K_d'V_i$ and $V_o + K_d''V_i$, respectively. When elution by solvent is recommenced the solutes cease to appear in the

effluent after the volume indicated in Fig. 7. It is apparent that for complete separation of the two solutes the volume of the sample should not be too large, i.e.

$$V_s < (K_d'' - K_d')V_i$$

This relation is valuable for a rough calculation of the column size necessary for given separation. For example since the K_d for NaCl is 0.9, V_s must be smaller than 0.9 V_i when desalting a protein ($K_d = 0$) on Sephadex G-25. Since V_i for this gel type is about 50 per cent of the bed-volume the maximal sample volume is 45 per cent of the bed volume. A separation in which this value was approached has been described (Flodin, 1961).

In a separation governed entirely by a molecular sieve mechanism the K_d-value cannot exceed unity. When greater K_d-values are found sorptive forces are operative as has been found for many substances. This will be discussed in more detail in chapter 9.

Non-ideal conditions

In practice ideal conditions for column operation are never attained and there are always some distortions of the ideal profiles. Three factors contribute: (1) local non-equilibrium, (2) longitudinal diffusion, and (3) perturbations due to irregular flow. A solute requires a certain time to diffuse into the gel particles and then out again, that is the diffusion equilibrium is not attained instantaneously and a local non-equilibrium arises. For a close approach to instantaneous equilibrium the gel particles would have to be very small but this is impractical due to the exceedingly low flow rates which would result. A compromise is always therefore necessary as concerns the particle size. Longitudinal diffusion is, generally, of little importance in the time required for a gel filtration experiment. Imperfect packing leading to irregular flow is practically the only important cause of deviation from ideality with a non-penetrant solute. For a penetrant molecule its effect is additive to the profile distortion resulting from local non-equilibrium. Both the theoretical and practical aspects of these phenomena have been studied intensively in ion exchangers (Helfferich, 1959). Glueckauf (1955) has derived an expression for the column behaviour of solutes with linear sorption isotherms in ion exchange resin gels. This should be valid also for gel filtration since only the distribution coefficient, diffusion coefficients in solution and in the gel, the flow rate, and the particle size are represented in the equations. It was shown that the EHTP (equivalent height of a theoretical plate) depends upon three terms, one of which represents imperfections in packing and longitudinal diffusion in the column and the other two, to film diffusion and gel diffusion, i.e. to local non-equilibrium.

The theoretical plate concept. In distillation it is a common practice to measure the efficiency of a column by calculating the EHTP. This concept was first applied to chromatography by Martin and Synge (1941) and in the last few years it has become widely used, especially in gas chromatography. For elution profiles having the shape of Gaussian error curves the number of theoretical plates can be calculated by the simple formula derived by Glueckauf (1955):

$$N = 8 \left(\frac{V_e}{\beta} \right)^2 \quad (1)$$

where β is the width of the elution curve at the height $C_{max}/2.72$ over the base-line. The EHTP is obtained by dividing the column length (l) with the number of theoretical plates obtained

$$EHTP = \frac{l}{N} \quad (2)$$

EHTP-values on Sephadex columns have been determined in relation to variations in elution rate and particle size (Flodin, 1961). Figs. 8 A and B show some elution curves for serum albumin in the porous gel Sephadex G-200 under varying elution rates and particle sizes. In table 8 the values of EHTP as determined from these curves are given. The results from both sets of determinations show a dependence on these variations similar to those obtained

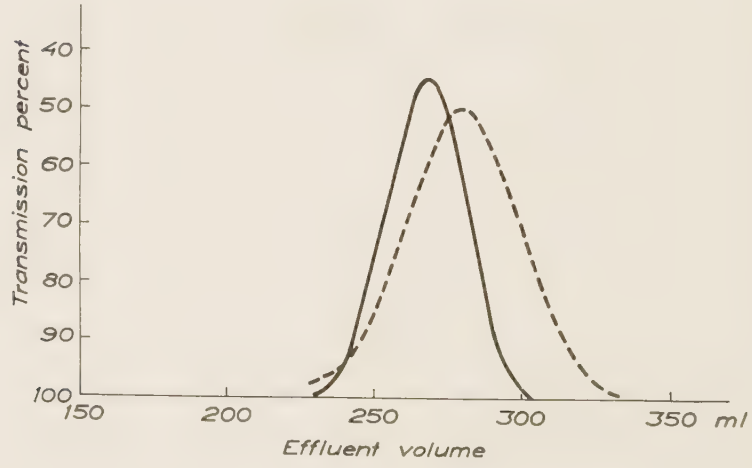


Fig. 8 A.

Elution diagrams of serum albumin from columns packed with two particle size fractions of Sephadex G-200. Solid line: the 200—270 mesh fraction; broken line: the 100—200 mesh fraction. Elution rate: 25 ml per hour.

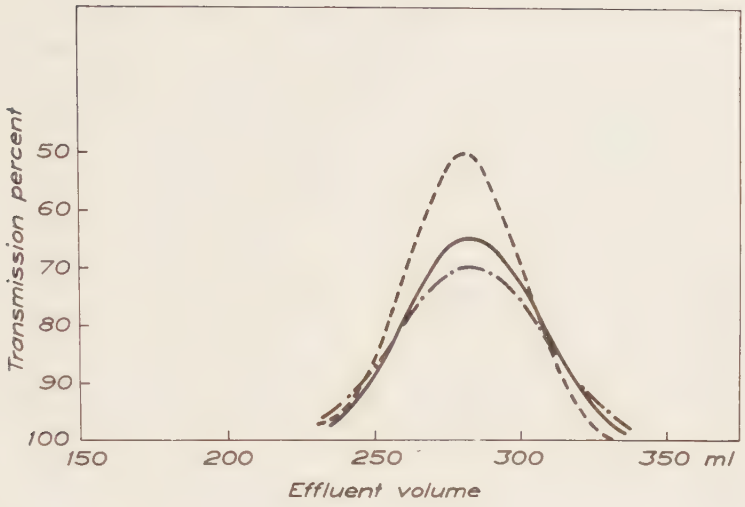


Fig. 8 B.

Elution diagrams at varying elution rates for serum albumin from columns packed with the 100—200 mesh fraction of Sephadex G-200. Elution rates were 25 (- - -), 50 (——), and 75 (—.—.) ml per hour.

for ion exchange resins. It should be observed, that for the protein, the EHTP obtained is much higher than those for the low molecular weight substances, when linear flow rates are compared. It reflects the lower diffusion constant of the protein.

Table 8.

EHTP values for serum albumin in a Sephadex G-200 column (4 cm × 42 cm)

Particle size mesh	Flow rate ml/hour	EHTP cm
100—200	75	0.62
100—200	50	0.48
100—200	25	0.23
200 -270	25	0.08

The most drastic effect on EHTP is obtained by variation of the particle size. It is therefore always desirable to perform an experiment with as small particles as possible. As mentioned above the limitation to this is the decrease in flow rate which occurs in columns packed with fine particles. Table 9 gives EHTP values obtained for a number of substances with differing molecular weights in Sephadex of various types and particle size fractions.

Table 9.

EHTP values obtained in various Sephadex types.

Type	Sieve fraction, mesh	Solute	Column dimensions cm × cm	Flow rate ml/hour	EHTP cm
G-25	50—80	uridylic acid	2 × 65	24	0.072
G-25	50—80	„ „	2 × 65	51	0.15
G-25	50—80	„ „	2 × 65	190	0.55
G-25	140—200	„ „	2 × 65	10	0.010
G-25	140—200	„ „	2 × 65	51	0.021
G-25	270—400	„ „	2 × 67	24	0.011
G-25	50—80	hydrochloric acid	2 × 65	24	0.069
G-25	270—400	„ „	2 × 70,5	24	0.026
G-25	50—100	glycine	3.5 × 39.4	120	0.064
G-25	200—400	„	1.8 × 49	18	0.011
G-25	200—400	glucose	4.5 × 150	30	0.035
G-25	200—400	cellobiose	4.5 × 150	30	0.034
G-25	200—400	cellotetraose	4.5 × 150	30	0.029
G-50	100—200	isoleucine	3 × 74	24	0.031
G-75	100—200	lysozyme	2 × 36	36	0.36
G-100	200—400	albumin	3.8 × 40	160	0.08
G-200	100—200	„	4 × 42	50	0.48
G-200	200—270	„	4 × 42	25	0.08

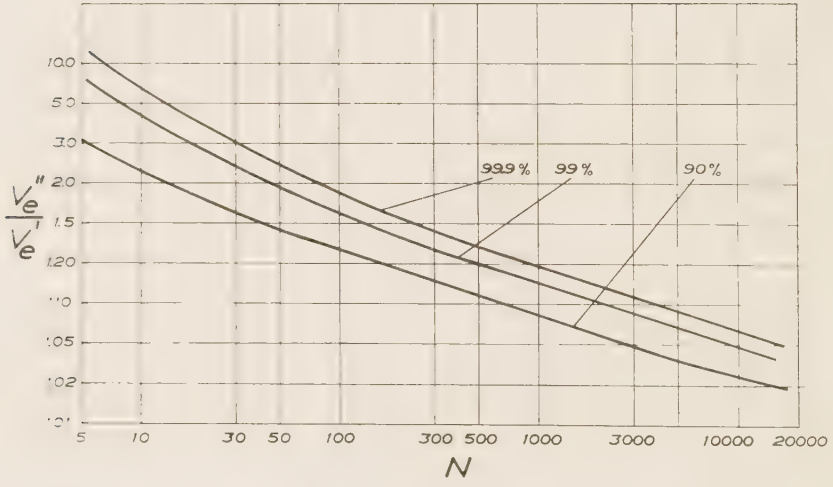


Fig. 9.

The relation between the ratio V_e''/V_e' and the number of theoretical plates necessary to obtain 90, 99, and 99,9 % separation of two solutes present in equal amounts. The elution curves should have the form of Gaussian error curves and the elution volumes are V_e'' and V_e'. (Calculated after Glueckauf, 1955).

Separability. The zone spreading has a pronounced influence on the resolution obtainable in a column. A method for the estimation of particle size, column size and flow rate necessary for a given separation has been described by Glueckauf (1955). He calculated the number of theoretical plates necessary to obtain a separation between two bands of Gaussian shape and containing equal amounts of solutes, for various degrees of overlapping. In Fig 9. the relation between the number of theoretical plates and the ratio V_e''/V_e' between the elution volumes is given, where

$$\frac{V_e''}{V_e'} = \frac{\alpha + K_d''}{\alpha + K_d'}$$

Here α is the fraction V_o/V_t. A few calculations will serve to illustrate the use of the diagram for the estimation of the smallest column size necessary for a given separation. The fraction α is generally in the range 0.25–0.80 and the value 0.30 will be used in the following calculations. In removing salts (K_d=0.9) from proteins (K_d=0) the ratio V_e''/V_e' becomes 4. A 99 per cent separation requires 15 theoretical plates. In table 9 it can be seen that Sephadex G-25, 50—80 mesh, gives EHTP values of 0.1 cm. This means that a column 1.5 cm long would be required for a 99 per cent removal of salt from a protein. If less dilution is desired a finer particle size fraction can be used and thus a shorter column. Sucrose and glucose have K_d-values of approximately 0.7 and 0.8, respectively, in Sephadex G-25. The ratio V_e''/V_e' becomes 1.1 and the number of plates necessary for a 99.9 per cent separation is 3,000. From equation 2 and table 9 it is apparent only particle size fractions finer than 200 mesh can be used with a reasonable column size. Thus, the 200—400 mesh fraction would require a column length of 90 cm.

CHAPTER 5

Experimental technique

Compared with many other chromatographic processes the elution volumes in gel filtration are small and thus separation depends to a large extent on the physical properties of the packed column. A zone should pass through the column as a narrow band without distortion. Careful pretreatment of the gel and packing of the column are therefore required. Once packed, a column can be used for long periods without repacking. Not unusually columns have been used for a year.

The choice of gel type and particle size fraction was discussed in chapter 4. Irrespective of type or particle size the gels can be handled in about the same manner in spite of large differences in density and the treatment described below will be generally suitable.

Sieving and swelling.

In some cases it is necessary to use fractions with a narrower distribution of particle size than are commercially available. The easiest way to obtain these is to sieve them dry in a laboratory sieve. All fractions used in this study have been treated in this way and the sieve fraction figures given refer to the U.S. Standard screen series. The finest fractions, however, are difficult to sieve

Table 10.

Swelling factors and approximate flow rates i 50 cm columns (pressure head 1 m) for Sephadex types and sieve fraktions.

Type	Sieve fraction mesh	Water regain g/g	Swelling factor ml/g	Flow rate ml/cm² hour
G-25	50—100	2.5	5	60
G-25	100—200	2.5	5	30
G-25	200—400	2.5	5	3.0
G-50	50—100	5.0	10	30
G-50	100—200	5.0	10	5.0
G-75	100—200	7.5	14	3.0
G-100	200—400	10.0	20	10
G-200	100—200	20	40	6.5
G-200	200—270	20	40	3.5

efficiently, due, among other factors, to electrostatic charge effects. To obtain narrow fractions finer than 200 mesh, hydraulic classification, for example in the manner described by Hamilton (1958), is recommended.

As an approximate guide for the amount of Sephadex needed for a particular column size the swelling factors as given in table 10 can be used. These were determined by letting a known amount of dry gel sediment in a measuring cylinder and reading the volume of the sediment. The figures are therefore somewhat higher than for a packed bed. An excess of about 20 per cent will generally be enough to compensate this and also losses due to removal of fines by decantation.

The gel is swelled by pouring it into an excess of water preferably with stirring to avoid clump formation. To avoid aggregation there should be some salt in the water (0.1—1 per cent sodium chloride) or the buffer to be used in the experiment. The swelling proceeds very quickly in the early stages due to the high affinity for water. For Sephadex G-25 and G-50 the final equilibrium is reached in a few hours. The types with lower swelling pressure, namely G-75—G-200, require longer times for equilibration and they should be allowed to swell for at least 24 hours at room temperature. If this is neglected the gel particles may continue to swell after packing into a column and a lowered flow rate or even complete stoppage of the column may result.

While the gel is being swelled it is suitable to remove fines by stirring up the gel and then allowing it to sediment. When a sharp boundary layer has formed the supernatant is decanted off. This process is repeated until the supernatant is clear. Four to five treatments usually give satisfactory results with a supernatant volume 10—20 times that of the gel. The times required depend on the type and particle size fraction of the gel and also on the dimensions of the vessel used. The bead polymerizates, G-100 and G-200, contain less fine material than the block polymerizates and can often be used without decantation. Before packing the gel suspension is diluted until its viscosity is such that air bubbles can rise rapidly through it. This is done to avoid clumps and trapping of air bubbles during packing.

Packing the column.

A cylindrical glass tube arranged in the manner shown in Fig. 10 is suitable for gel filtration in columns with a diameter of 4 cm or less. To avoid dilution of the zones during removal from the column, special care has to be taken with the outlet. A small piece of glass wool is laid on the outlet capillary and then a layer of 0.1 mm glass beads. In experiments with blood serum containing lipoproteins these were exchanged for cottonwool and Sephadex G-25 Coarse particle size respectively. For columns with diameters of 5 cm. or larger an

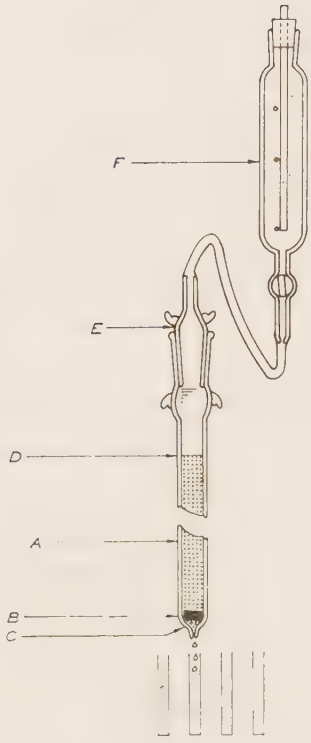


Fig. 10.

Arrangement for gel filtration. In the column (A) a piece of glass wool (C) is placed on the outlet capillary, and above it a layer of tiny glass beads (B) on which the gel bed rests. The bed surface is covered by a circular filter paper (D). Through the joint (E) an eluant reservoir (F) is connected.

arrangement similar to that described on page 76 for automatic operation is more suitable.

A number of different techniques for packing the column have been investigated. A method giving good result with Sephadex G-25 and G-50 has been described (Flodin, 1961). A simpler method, useful for all Sephadex types, and giving very good results will be described here. The column is carefully mounted vertically and the outlet closed. It is filled to one third with water or buffer and the glass wool and the glass beads are then put in place. An extension tube is connected. The column and the tube is now filled with the gel suspension which immediately starts to sediment. When a layer of 2—5 cm has formed the outlet is opened and a slow stream of water allowed to flow out. A horizontal zone of packed material soon becomes visible and allows visual control of the process. If the zone becomes skew the packing should be interrupted. As soon as the extension tube is devoid of gel the supernatant is removed and more suspension added. This is repeated until the desired bed length has been obtained. It is essential that new suspension is added before all gel previously present has packed.

After packing, the extension tube is removed and the top layer of the bed stir-

red up. The column is then connected to a reservoir containing the eluant to be used and washed until the bed is stable. At least 3—5 bed volumes should be used. Before addition of the sample it is often necessary to stabilize the bed surface mechanically. For this purpose a well-fitting circular disc of filter paper or porous polyethylene has been successfully used. For larger column diameters this is not satisfactory but a short plastic cylinder with a fine-mesh stainless steel or nylon net of the same type as that used for the distributor in the apparatus in Fig. 24 of chapter 10 can be used.

Technique of gel filtration.

Loading the sample. Two methods have been used with good results. In the first, the eluant above the bed is removed and the sample carefully placed on top of the bed and in the second, the sample is layered between the bed and the eluant. It is necessary for the density of the sample to be higher than that of the eluant in the latter method.

Method 1. In this method it is necessary to protect the surface in the manner described in the preceeding paragraph. When the supernatant has been removed the sample is carefully added with a pipette. The bottom outlet is then opened and the sample allowed to enter the bed. At the moment it disappears through the surface a small amount of eluant is added to wash the surface and when this has disappeared a larger volume of eluant is added. The eluant reservoir is then connected and elution started.

Method 2. In this method no protection of the bed surface is necessary and it has proven especially valuable for blood serum protein separations on Sephadex G-200, since the lipoproteins often clogged filter paper or porous plastic plates. The addition of the sample must be done carefully to minimize dilution and avoid disturbance of the gel and can conveniently be accomplished with a motor driven syringe. A thin plastic tube connected to the syringe is carefully positioned in the supernatant a few millimeters above the bed and the motor started. In this way a 20 ml sample was added in seven minutes to form a narrow zone between the bed and the eluant above. After sample addition, the plastic tube is removed, the column connected to the reservoir and the elution started.

If the density of the sample is too low it may be adjusted by adding a denser non-disturbing substance. When blood serum is eluted with molar sodium chloride, addition to the serum of sodium chloride or sucrose has given excellent results. In all Sephadex types these additives soon become separated from the proteins and do not influence either the separation or the analysis.

Elution. Zone spreading depends to a considerable extent upon the elution rate which must be controlled. In columns packed with fine particle size fractions of Sephadex this is no problem, since only very low flow rates are obtainable. Usually the elution is carried out under a constant hydrostatic pressure head. This can be done by the simple arrangement of Fig. 10, which gives a constant flow rate provided the sample viscosity is not appreciably different from that of the eluant. When using constant volume pumps care must be taken to avoid excessive pressures on the top of the column. In Fig. 26 on page 78 some pressure versus flow rate curves are shown. It is evident that in the steep region even small flow rate increments can give rise to very high pressures.

For the calculation of elution volumes it is useful to collect the effluent from the column in a volume operated fraction collector. In many experiments reported here, however, a constant flow rate and a time-operating fraction collector were used. A convenient arrangement for routine separations utilizing a syringe to deliver predetermined volumes was described by Östling (1960).

Regeneration. A characteristic feature of the gel filtration method is that in general all substances present in the sample are eluted without change of eluant. Thus the column is regenerated during the experiment and the bed can be used for a large number of consecutive experiments (compare chapter 10). Clogging due to dust particles present in the eluant or precipitation of some components in complicated mixtures cannot, however, be avoided. The bed can be regenerated, however, by removing the gel and letting it sediment a few times, in water, or the eluant. The impurities which diminish the flow rate tend to remain in the supernatant and can be decanted away. This has proved to be an excellent way of recovering gel beds which have seemed completely unusable.

In larger columns it is more practical to use backwash for the removal of solid impurities. Water or the eluant is introduced through the bottom of the column, to which an extension tube has been added. With large particle size fractions the bed expands easily but especially with small gel particles it may move upwards as a plug which can be disintegrated by inserting a thin rod through the bed. The upward flow rate is then adjusted until the upper boundary of the expanded bed is well below the top of the extension tube. The process is continued as long as the overflow is turbid. On cessation and reversal of the flow the column can be repacked.

Checking the packing. The importance of a good packing has been emphasized already many times. It is essential to check the state of the bed and the beginner especially needs a good method for doing this. Much valuable information is obtained by passing a coloured substance of high molecular weight through the bed. It is possible to see where an irregularity distorts the zone and also its effect. In previous publications India ink has been recommended in salt

free beds and hemoglobin when electrolytes are present. In general, any coloured high molecular weight substance can be used; this should work preferably over the whole pH range and be unaffected by the electrolytes present. Recently coloured dextran derivatives have been produced and these seem ideal for this purpose. In a previous publication (Flodin, 1961) a rule of thumb has been given, namely that in a well packed bed a completely excluded substance added in a volume corresponding to 10 per cent of the total bed volume should appear in the effluent in a volume not exceeding double that of the sample.

Sterilization and preservation of the bed material. Sephadex can be sterilized in swollen condition by treatment at 110 C for 40 minutes in an autoclave without appreciable changes in properties or loss of material. Matheka and Wittmann, (1961) have investigated sterilization with formaldehyde. By passing a solution of the agent through packed Sephadex, sterility could be obtained without affecting the separation properties.

To avoid microbial growth in columns kept for long time intervals, the addition of some preservative to the eluant is valuable. Toluene, phenol or cresol have been used successfully but generally buffers saturated with chloroform are used in this laboratory. Tris buffers can be used for long times without any additive.

Determination of distribution coefficients.

As mentioned in chapter 4 the distribution coefficient for a solute can be expressed as follows:

(1) $$K_d = \frac{V_e - V_o}{V_i} = \frac{V_e - V_o}{a\ W_r}$$

where V_e, V_o and V_i are the elution volume, the void volume, and the imbibed volume, respectively. W_r is the water regain and a is the amount of dry gel present in the column. The K_d-value can also be determined as follows (Granath and Flodin, (1961):)

(2) $$V_t = V_o + V_i + V_g$$

where V_t and V_g are the total bed volume and the volume of the gel matrix, respectively. From the definition of the water regain it follows that

(3) $$V_i + V_g = \frac{a}{d}\ (W_r + 1)$$

where d is the density of the swollen gel (for determination see page 31) which can also be calculated from its water regain and dry density (1.64). Combination of equations (1), (2) and (3) gives

$$K_d = \frac{1}{a\,W_r}\left[V_e - V_t + \frac{a}{d}(W_r + 1)\right] \quad (4)$$

The determination of the parameters necessary for the calculations will be described below with the exception of the density and water regain, which were treated on page 31.

The total bed volume is best determined by calibrating the column with known volumes of water.

The elution volume V_e is the volume of eluant required to move the peak concentration of a component from the top to the bottom of the bed. If the effluent volume is measured from the moment the sample enters the bed, the volume of the peak concentration in the elution diagram represents the elution volume provided corrections are made for the dead volume below and in the outlet from the column.

The void volume V_o is the elution volume for a solute known to be completely excluded from the gel particles. For this purpose India ink can be used for all gel types in the absence of electrolytes. Narrow fractions of dextran with molecular weights sufficiently high to be excluded from the particular gel type used in the column are useful as are many other substances with known properties.

The imbibed volume V_i is equal to the product $a \cdot W_r$, when the density of water is assumed to be unity. Since small amounts of fine gel particles are removed in the sedimentations preceding packing, the weight of dry gel is best determined after the gel filtration experiments. The gel is then dehydrated with ethanol and dried as described on page 17. In order to correct for a small residual amount of ethanol trapped in the gel, an aliquot of the dry gel is taken, moistened with water and dried to constant weight at 105°C.

The sum of the imbibed volume and the void volume ($V_o + V_i$) can be determined in an elegant manner by measuring the elution curve for tritiated water (THO), which is known to penetrate the gel to the same extent as ordinary water (Marsden, personal communication). Solutes with K_d-values known to be near to that of THO can also be used. Thus, KCl (K_d 0.98 in Sephadex G-25) gives values sufficiently accurate for most purposes.

CHAPTER 6

Desalting and group separation

The removal of salts and small molecules from solutions containing macromolecules will be treated in this chapter. Due to the large difference in distribution coefficients this type of separation can be made easily with very simple columns and with high flow rates. In this respect gel filtration compares favourably with dialysis, by which procedure similar separations can be obtained.

Large molecules do not penetrate dextran gel particles and have K_d-values of zero. This means that for Sephadex G-25, solute molecules with molecular weights higher than about 5,000 are eluted in the void volume. On the other hand salts and many molecules with molecular weights below about 1,000 have K_d-values in the range 0.7—1. From the discussion on page 41 it is apparent that columns with relatively few theoretical plates suffice for complete separation. Thus, for a difference in K_d-values of 0.9 only 15 theoretical plates are necessary for a 99 per cent separation. This modest requirement is easily achieved with any one of the particle size grades available. The particle size chosen will influence other factors, such as the duration of the experiment, the dilution of the zones etc.

Because of the high flow rates obtainable with coarse particles (50—100 mesh) these are generally preferred for experiments in which large sample volumes are separated. This will be discussed further in chapter 10. For sample volumes not exceeding a few hundred milliliters smaller particles (100—200 mesh) are convenient, since the experimental time is still short and better resolution together with less dilution is obtained. The linear flow rate for 100—200 mesh particles is about 0.5 cm per minute, which allows a complete experiment in 20 minutes with a 10 cm column and in 3—4 hours with a 100 cm column.

It is evident, that in order to make an experiment rapidly, short columns should be used. However, for good resolution the ratio of diameter to height must not be too high. In this laboratory good results have been obtained with a ratio of 1: 5 although 1: 10 to 1: 20 are ratios more frequently used.

Some of the factors influencing separation have been discussed (Flodin, 1961). It was concluded that for minimal dilution of the zones the sample volume should be large relative to the bed volume. The maximal theoretical value is about 45 per cent of the bed volume and in an experiment with a dilute hemoglobin solution this value was approached (Flodin, 1961). In practice such high

efficiencies are generally not obtained and usually a sample volume of 15—25 per cent of the bed volume is the maximum which can be used.

The concentration of the sample is itself of minor importance but severe disturbances in the elution pattern are obtained if the viscosity is too high. Increase in viscosity leads, in the first place, to a reduced flow rate and also to irregular flow patterns inside the column and a decreased efficiency. A relative viscosity of 10 centipoises was too high for a complete separation of sodium chloride from hemoglobin when the sample volume was 10 per cent of the total volume (Flodin, 1961). High colloid concentrations exert osmotic pressures, which may cause shrinkage of the gel with consequent disturbances in the flow pattern. Under the conditions studied in this laboratory osmotic phenomena have never been observed to produce severe disturbances.

Since the gel particles act as membranes impermeable to high molecular weight polyelectrolytes Donnan effects can occur. Small electrolytes are then redistributed so that there will be a higher concentration inside the gel particles. When desalting polyelectrolytes the concentration of small ions in the zone containing the macromolecules decreases and the Donnan effect becomes more pronounced. A sharper boundary between the polyelectrolyte and the low molecular weight electrolyte is thus created and the separation is improved.

Desalting.

The realization that dextran gels could be used to achieve rapid desalting of protein solutions gave the impetus to the development of the gel filtration method. In the first publication the separation of a serum albumin fraction from the large amount of ammonium sulfate used to precipitate the globulin fraction was described (Porath and Flodin, 1959). Since then there have been numerous publications about desalting by gel filtration. Only a few typical applications will be considered here. Ringertz and Reichard (1960) separated milligram amounts of acid polysaccharides including heparin from salt in 10 minutes. Similarly, a polysaccharide from mast cell tumours was also desalted (Ringertz, 1960).

In a procedure for the isolation of haptoglobins Cornell and Shaw (1961) removed salts from serum by passage through Sephadex G-25 before fractionation by ion exchange. After adjusting the pH of the serum to 4.6 it was filtered through a bed (9 cm $\times$ 60 cm). In each experiment 500 ml of serum were added and collected in the effluent in 600 ml which corresponds to a dilution factor of 1.2. Some proteins were probably precipitated on the Sephadex, since it had to be removed and decanted for regeneration after 6 to 8 runs.

Due to the poor solubility of many proteins in the absence of electrolytes, precipitation may occur during the passage through a column. Sometimes the

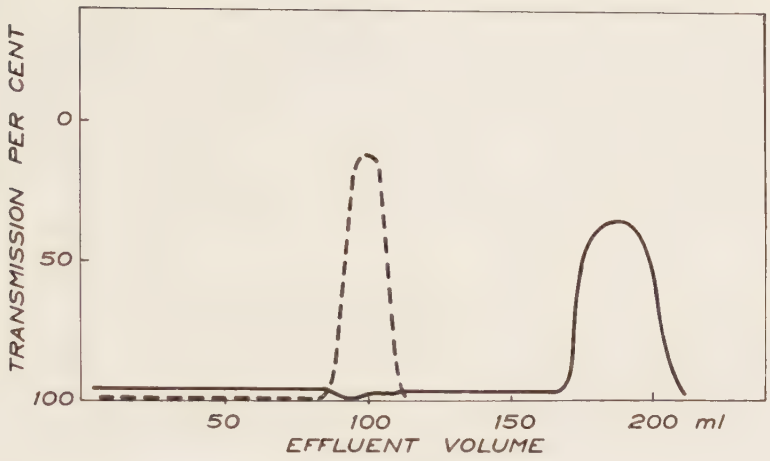


Fig. 11.

Diagram illustrating desalting of a protein solution. The broken line (- - -) indicates the UV-absorption as recorded with a Uvicord and the solid line (——) the conductivity as recorded with a Conductolyzer.

precipitates are not eluted but stick to the gel particles, ruining the experiment. Complete desalting is, however, possible even in such cases by an indirect procedure. The protein is first transferred into a volatile electrolyte system by passing it through a Sephadex column prewashed with the volatile electrolyte solution. The salt is then removed by lyophilization. The precipitation can also be utilized for fractionation (see below).

A frequently encountered step in the purification or characterization of proteins is the transfer to another electrolyte environment. This is usually done by dialysis against the new electrolyte solution. With gel filtration the procedure is the same as with desalting and is made in a column prewashed with the new electrolyte solution which is also used as the eluant. The process has been studied for solutions of CO-hemoglobin and it was observed that when the pH is to be changed a smaller sample volume than in ordinary desalting may be necessary. In particular this is the case with high protein concentrations and a low buffering capacity of the new buffer system (Flodin, 1961). In Fig. 11 such a separation is illustrated. The protein originated from a serum fractionation on Sephadex G-200 and was afterwards further fractionated by ion exchange.

An interesting application of the desalting procedure has been described by Matheka and Wittmann (1961). They found that a number of virus suspensions could be freed from salts by gel filtration with smaller losses of activity than by dialysis. This presumably depends on the short duration of the experiment.

The removal of radioisotopes from proteins has been reported. When using I^{131}-albumin for plasma volume determinations the presence of free I^{131} may

cause considerable errors. By filtering the labelled albumin through gel immediately prior to use the free iodide content could be almost eliminated (Bill et al., 1960). Spitzy et al. (1961) studied the conditions necessary for optimal separation and found that an eluant containing salt should be used since in distilled water or very low salt concentrations I^{131} was excluded from the gel phase (cf. chapter 9). Chromate-Cr^{51} could also be separated from a labelled hemoglobin (Bill et al., 1960).

Removal of by-products in reactions with macromolecules.

The removal of small organic molecules has been reported in many papers. Thus, the separation of glucose from dextran was one of the first studied (Flodin and Porath, 1959; Östling, 1960; Bill et al., 1960). In this laboratory gel filtration has been found an excellent means of removing by-products after substitution reactions of macromolecules. For example, in the reaction between dextran and chloroacetic acid in the presence of sodium hydroxide to form carboxymethyl dextran, sodium chloride, glycolic acid and diglycolic acid are formed as by-products, which can all be removed by a single passage through a Sephadex G-25 column. Similar experiments with proteins have been described by Anfinsen and Haber (1961). They passed reduced ribonuclease through a Sephadex column to remove mercaptoethanol, urea and salt. The method was also applied to some other proteins.

In periodate oxidation of biopolymers the low molecular weight reaction products were removed by passage through Sephadex G-25. Since periodate attacks the gel (cf. page 28) the excess was reduced before the gel filtration step. (Chatterjee et al., 1961.)

Antibodies marked with fluorescent dyes are used extensively in immunology. The removal of unbound dye after the substitution is essential and has generally been performed by lengthy dialysis. The treatment can be shortened from 7 days to less than an hour with gel filtration (Killander et al., 1961; Zwaan and van Dam, 1961; George and Walton, 1961, Folkergill and Nairn, 1961; Curtain, 1961; Lipp, 1961). An important consequence of the rapid removal was the much higher activities obtained with some antibodies.

Group separation.

A typical example of group separation is the removal of amino acids, peptides, glucose etc. from serum proteins (Porath, 1959). In the isolation of keratosulfate from chondromucoprotein Sephadex G-75 was used to remove oligosaccharides from a hyaluronidase digest of the protein. After further degradation with papain a passage through Sephadex G-50 served to remove

amino acids and peptides (Gregory and Rodén, 1961). The further fractionation of the oligosaccharides from the first gel filtration step is described in chapter 7.

Cofactors for methionine-synthesizing systems from E. coli mutants were completely removed by gel filtration but only partially with dialysis (Kisliuk, 1960). The soluble fraction from the mammary glands of lactating rats gave higher rates of a adenosine triphosphate-pyrophosphate exchange after gel filtration (Bucovaz and Davis, 1961). Soluble ribonucleic acid could be purified efficiently on Sephadex G-50 but could not be separated from ribosome ribonucleic acid even in Sephadex G-75.

A group separation of a yeast extract was reported to give five peaks (Gelotte, 1961). The first contained nucleic acid and polynucleotides and the second oligonucleotides. A large number of low molecular weight components were identified in the third peak. The slowest peaks contained components which had adsorbed to the gel and contained adenylic acid and nuceosides, respectively. Separations of a similar character were reported by Zadrazil et al., (1961).

Salting-in and salting-out.

The precipitation of proteins due to removal of salts mentioned above can be used to effect separations. At an early stage of the development of the gel filtration method, the author studied the fractionation of serum proteins by passage through a Sephadex G-25 column equilibrated with low ionic strength buffers. Under salting-in conditions the proteins appeared in two peaks, the first containing dissolved compounds and a precipitate. The second component was precipitated on the surface of the gel particles and redissolved when the salt originally present in the sample passed over it. The recovery of the proteins of the second peak could be increased by the introduction of a zone of e.g. 0.5 molar sodium chloride immediately after the sample. Fig. 12 illustrates a separation of serum albumin and γ-globulin.

A similar method has been used to separate complement factors (Fjellström, 1962) in a column equilibrated with acetate buffers at pH 5.1—5.6. The protein peak eluted first contained in addition to soluble proteins precipitated euglobulins. The factor C'1 was entirely in the precipitate while the factor C'2 remained in solution. Experiments performed under carefully controlled conditions were highly reproducible and allowed rapid production of complement-reactive sera.

Epstein and Tan (1961) used 0.02 M phosphate buffer at pH 8.0 to elute a serum sample containing 4 per cent sodium chloride and separated pseudoglobulins and euglobulins. They obtained two peaks which were characterized by zone electrophoresis. The slower peak consisted almost entirely of γ-globulin. With sera from rheumatoid arthritis patients the rheumatoid factor appeared in this fraction.

By introducing a gradient, rising in the direction of flow, of a low molecular weight protein precipitating agent, separation by fractional precipitation can be obtained (Porath, personal communication). A protein sample introduced into the column migrates faster than the agent and thus comes to a region where precipitation occurs. As the gradient migrates conditions become suitable for redissolving a particular protein component which travels downward again only to become reprecipitated once more and so on. This principle is applicable to fractionation of serum proteins. To obtain the best results the method requires careful design of the experiment with special attention to the column size and elution rate.

Complexes.

Complexing reactions can be utilized in gel filtration. This principle was first utilized by Lindner et al. (1959) for the purification of oxytocin and vasopressin. An extract at pH 5.9 from posterior lobes of hog pituitaries yielded the hormone activities in the "non-penetrant" peak. After dissociation with molar formic acid the hormone activities penetrated the gel particles and appeared in the effluent after the main part of the material. A very efficient and rapid purification of the two hormones could thus be obtained in two steps.

A very interesting example of the use of complex formation for isolating

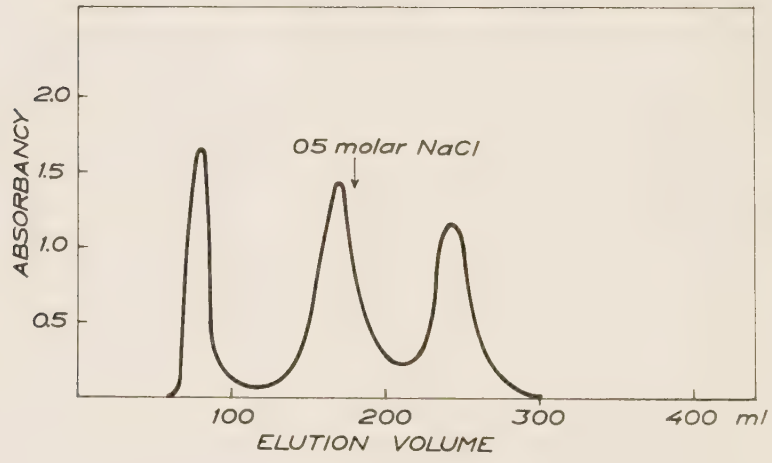


Fig. 12.

Elution pattern of a mixture of serum albumin and γ-globulin added to a column of Sephadex G-25 prewashed with distilled water. The 0.5 molar NaCl solution used for elution appeared in the effluent at the arrow. The third peak is a non-protein component present in the albumin preparation.

vitamin B_{12} from sea water has been described by Daisley (1961). The vitamin and salt appear together when passed through Sephadex G-25, but upon addition of an "intrinsic factor" preparation, a complete separation of the complexed vitamin from salts could be obtained, allowing an assay of the vitamin content.

Concentrating with Sephadex.

Sephadex may be used for concentrating macromolecular solutions. This is not a chromatographic procedure but depends on the molecular sieve properties of the gels. When dry Sephadex is added to a solution of non-penetrant molecules it swells and thus removes water from the solution (Flodin et al., 1960). Since low molecular weight solutes have K_d-values of the same order of magnitude as that of water, changes in their concentrations are very small. In the liquid surrounding the gel particles the macromolecules are present in concentrated form while the buffer ionic strength and pH is not appreciably changed.

The procedure is performed by adding a calculated amount of dry Sephadex, letting it swell and then separating the liquid in the interstices from the gel particles by an efficient method such as centrifugal filtration. A two or three fold concentration can thus be obtained per step. The most suitable gel for this purpose is Sephadex G-25, Coarse, due to its rapid swelling and the ease of separating it quantitatively from the supernatant. Presumably, the procedure is most useful for concentrating sensitive macromolecules on a rather large scale.

CHAPTER 7

Fractionation of oligosaccharides and polysaccharides

In a homologous series the difference in distribution coefficients between successive members are small, which means that columns with high efficiency must be used. For example, to separate isomaltose and glucose with K_d-values of 0.8 and 0.7, respectively, a column with at least 3000 theoretical plates is required if the fraction α is 0.3 (chapter 4). Even with small particle sizes rather long columns have to be used, so that the flow rates are very low, viz. the separations take relatively long times. In addition, relatively small amounts, compared with desalting procedures, can be separated in each experiment. In the preparation of a column great care should be taken in packing and it is recommended to check the efficiency before use by passing a similar test solute through the column to determine if the desired number of theoretical plates can be obtained. Once an efficient column has been obtained it can be used for very long periods without repacking if clogging or microbial growth are avoided. Thus, a column used for separating oligosaccharides has been used repeatedly for more than a year without deterioration.

Neutral oligosaccharides.

Oligosaccharides in the isomaltose series were chosen as one of the first objects to test the possibilities of the gel filtration method. It was soon found that only a partial fractionation could be obtained unless very fine particle size fractions were used. Nevertheless, useful prefractionations can be obtained with e.g. 100—200 mesh particles.

Fig. 13 illustrates an experiment in which oligosaccharides of the isomaltose series were separated in a 3 cm × 120 cm column packed with Sephadex G-25, 200—400 mesh, and eluted with distilled water. The flow rate was 20 ml per hour and the time for the experiment was 40 hours. The fractions were evaporated and the material identified by paper chromatography. The molecular weights were estimated from the number of reducing end groups, determined as according to Somogyi (Isbell et al., 1953), and found to correspond with those expected. The results have recently been confirmed (Granath, personal communication).

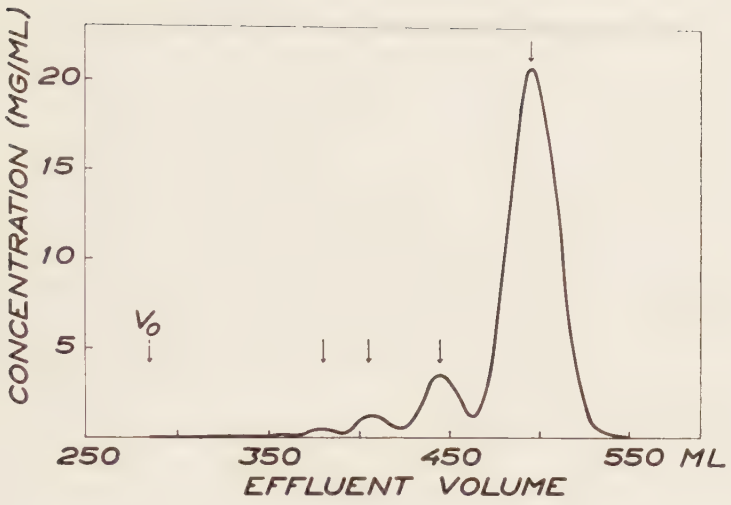


Fig. 13.

Separation of oligosaccharides of the isomaltose series in a column of Sephadex G-25, 200—400 mesh. Arrows indicate, from left to right, the void volume (V_o), isomaltotetraose, isomaltotriose, isomaltose, and glucose. The sugars were identified by paper chromatography.

Oligosaccharides in the cellodextrin series have also been separated (Flodin and Aspberg, 1961). After acetolysis and deacetylation the individual oligosaccharides up to the hexaose were isolated. They were identified by paper chromatography, molecular weight determination and X-ray diffraction. The only oligosaccharide showing significant amounts of impurities was the cellohexaose.

Acidic oligosaccharides.

When acidic oligosaccharides, derived from chondroitin sulfate by digestion with hyaluronidase, were eluted with distilled water the resolution was much inferior to that obtained with neutral oligosaccharides. The use of eluants containing salt, however, improved the separation and the higher the ionic strength the sharper were the peaks obtained. In Fig. 14 are illustrated two experiments made with a column 3 × 150 cm packed with the 200—400 mesh sieve fraction of Sephadex G-25 (Flodin, Gregory, and Rodén, to be published). In both experiments 10 ml of a 10 per cent oligosaccharide solution were used and the eluant was 0.1 and 1 molar sodium chloride, respectively. Resolution was not complete with 0.1 M NaCl. The fractions were analyzed with the carbazol method (Dische, 1947) and identified by paper chromatography as the

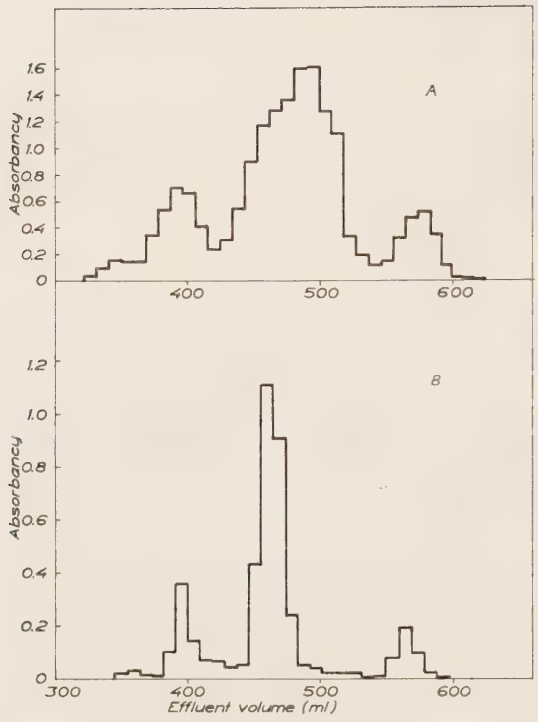


Fig. 14.

Elution diagrams of oligosaccharides, derived from chondroitin sulfuric acid, from a column (3 cm × 150 cm) of Sephadex G-25 (200—400 mesh.) Elution rate: 30 ml per hour. In diagram A the eluant was 0.1 molar NaCl, and in diagram B 1 molar NaCl. The material in the peaks was identified as the octa-, hexa-, tetra-, and disaccharides (from left to right). The most abundant component was displaced towards a higher elution volume in 0.1 molar NaCl.

di-, tetra-, hexa- and octasaccharides. It is interesting to note that digests of hyaluronic acid with hyaluronidase showed the same type of behaviour but with an excellent resolution already in 0.1 molar sodium chloride. Thus, there is a parallellism between the charge density, which is higher in the chondroitin sulfate oligosaccharides, and the ionic strength necessary for high resolution. It is probable that the failure to separate oligonucleotides with Sephadex (Sober, personal communication) is due to similar phenomena.

A reasonable hypothesis as to the cause of this effect is that high osmotic pressures act upon the "forbidden regions" defined in chapter 3. In the front of the peak the concentration is low, as is the osmotic pressure. This part of the peak will therefore migrate faster than the more concentrated portion which due to its osmotic pressure would extract water from the "forbidden region" and migrate at a slower rate.

Polysaccharides.

As is evident from the oligosaccharide separations reported the difference in K_d-values between individual successive members of a homologous series

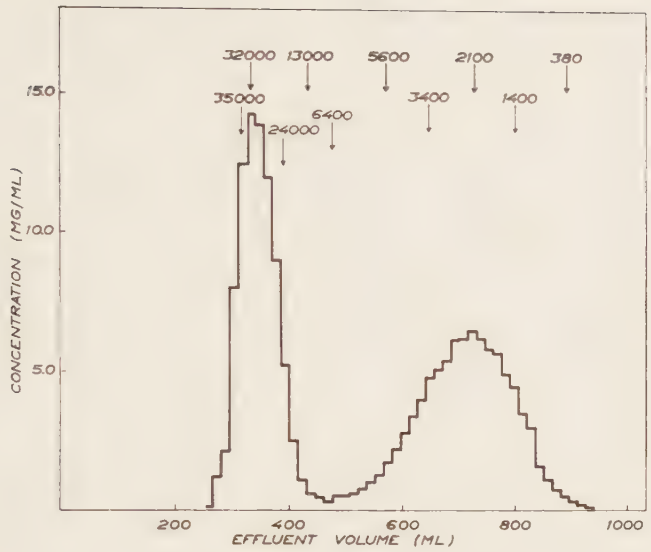


Fig. 15.

Elution pattern of a mixture of two dextran fractions from a Sephadex G-75 column. The arrowed figures indicate the number average molecular weights of the fractions.

becomes smaller the higher the degree of polymerization. The number of theoretical plates necessary for complete separation soon becomes so large that a column of high enough efficiency becomes unrealistic. But efficient fractionations leading to partial separations are still possible.

A number of dextrans with varying molecular weights have been subjected to gel filtration with distilled water as eluant and some results have been reported (Flodin and Granath 1959, Granath and Flodin, 1961). Depending on the molecular weight of the dextran, Sephadex types of different water regain values had to be used. Thus, G-25 gave fractionations up to a molecular weight of roughly 4000, G-50 up to 10,000, and G-75 up to 40,000. With the more porous gels Sephadex G-100 and G-200 it was possible to extend the range considerably (Granath, personal communication).

The results obtained compare favourably with fractionations obtained by extraction or careful precipitation. The latter two methods have an inherent weakness in the lower molecular weight regions in that the dextrans have a tendency to crystallize. If this occurs the result becomes quite misleading, since the solubility characteristics of a crystalline dextran differs radically from that of the amorphous form. In the gel filtration method the risk of crystallization is almost non-existent and the method is therefore preferable for molecular weights below about 50,000.

An example of a simultaneous fractionation of a mixture of two dextrans is shown in Fig. 15. The Sephadex type in this case was G-75. The heterogenity ratios ($\bar{M}_w/\bar{M}_n$) were determined for some fractions and found to vary from 1.16 to 1.34.

By plotting V_e/V_t, which is proportional to K_d, against the molecular weight it was established that there is a smooth change in K_d-values over the entire separation range, which extends down to very small molecules for all Sephadex types.

CHAPTER 8

Fractionation of peptides and proteins

The behaviour of peptides and proteins in gel filtration is largely governed by the same factors as with the neutral oligo- and polysaccharides. The same molecular weight limits can be used as a guide for the decision as to which Sephadex type is most suitable for a particular separation problem. However, due to protein-protein interactions and adsorption to the gel the situation in many cases is far more complicated. In order to obtain a separation based on size is necessary to take these effects into account. Since the molecular sieve properties of dextran gels are insensitive to the electrolyte environment, the eluant composition can be chosen almost at will. Thus, to minimize protein-protein interactions due to electrostatic forces the separation can be made in a high ionic strength medium or at a suitable pH-value. Interaction due to hydrogen bonding is eliminated by a medium containing urea or other hydrogen bond breaking agents. These are only a few examples of the many variations possible and further information can be obtained from the publications cited below.

The manner in which the different types of interactions influence separations is best illustrated by a few examples. In Sephadex G-25 the basic polypeptide viscotoxin (molecular weight 9,000) was excluded and appeared therefore in the effluent after the void volume. However, when passed through Sephadex G-50, into which it can penetrate, it was adsorbed and migrated at an even lower rate than the amino acid isoleucine (Porath, 1960). In an experiment with a commercial preparation of crystalline cytochrome C on Sephadex G-75 the enzyme, contrary to expectation, was completely excluded from the gel particles. When subjected to gel filtration in the presence of 0.2 molar acetic acid a purified product was obtained, which behaved normally in subsequent gel filtration at neutral pH-values (Gelotte, personal communication). These experiments show clearly the danger of drawing too far-reaching conclusions from single gel filtration experiments, especially when molecular weight estimations are to be made.

Part 1. *In Sephadex G-25, G-50, and G-75.*

A detailed study of the separation of peptides obtained after tryptic hydrolysis of α-casein has been made by Bennich (1961). He studied the separation on

Sephadex G-25, G-50 and G-75 under similar conditions. Four peaks were obtained on G-25 while with G-50 there were seven peaks with different contents of peptides as revealed by paper electrophoresis. In G-75 the resolution was not better than with G-50 but the trypsin activity which migrated with the void volume in the other types was split into two enzymatically active peaks. All experiments were made in molar acetic acid since the other media tried gave inferior resolution. The sample size and concentration as well as the elution rate could be varied within rather wide limits without diminishing the efficiency.

Fractionation of peptides obtained by tryptic digestion of casein has also been reported by Phillips and Gibbs (1961). They obtained four ninhydrin-reacting peaks when eluting with 0.2 molar ammonia. "Fingerprinting" of the fractions showed that effective separations had been obtained. The maximum growth stimulating effect for a strain of Streptococcus equisimilis was found in a fairly narrow region of the total peptide material. Only three peaks were revealed by UV-analysis at 280 mμ. The ninhydrin and UV-curves did not show correspondence, because of the small proportion of aromatic amino acids in the first peaks.

In their studies on diffusible peptides obtained by tryptic digestion of the α-chain of human hemoglobin, Hill and Konigsberg (1960) were able to resolve all peptides by a combination of counter-current distribution and gel filtration. They used 0.2 molar acetic acid as eluant and noted that not all peptides were separated on the basis of size. Eaker (1962) found that a tryptic digest of oxidized ribonuclease A could be resolved into three peaks in Sephadex G-25 using 0.2 molar acetic acid as eluant. As expected the fastest peak contained the largest peptides with 19—22 amino acid residues. The second contained tetra- and heptapeptides and the third not only di- and tripeptides but also one hepta- and one decapeptide. The two last-mentioned peptides each contained two tyrosine residues which are known to be adsorbed to Sephadex and thus may be the cause of the late appearance of these peptides.

For the preparative separation of peptides obtained by tryptic digests of tobacco mosaic virus it was found suitable to work in 0.01 molar sodium hydroxide (Stepanov et al., 1961) which allowed the separation of larger quantities than was possible in 0.02 molar phosphate buffer at pH 7.5. The peptides were separated according to size but measures had to be taken to prevent specific interactions between them. In one case separation was only obtained on Sephadex G-25 when the eluant contained 0.1 molar sodium phenolate. One peptide mixture could be resolved on Sephadex G-50 by adding 8 molar urea to the sample. The urea appeared as a separate peak after the peptides.

Kallidins are peptides produced by treatment of human plasma with human urinary kallikrein. In the isolation of these peptides two steps with Sephadex G-25 were used. In the first 0.002 molar hydrochloric acid was the eluant. The active fractions from this experiment were subjected to a further gel filtration in

0.01 M ammonium formate. The purification obtained as measured by the biological activity was 10 times in the first and 3 times in the second step (Pierce and Webster, 1961).

The separation of the ferriporphyrin-C peptide from the other products of peptic digestion of cytochrome C on Sephadex G-50 has been reported by Porath (1960). The purification of oxytocin and vasopressin by Lindner et al. has already been mentioned (page 54).

The selectivity of Sephadex G-25 is not high enough to distinguish between amino acids and the peptide hormones, vasopressin and oxytocin. In some mixed solvents, however, the swelling of the gel is less pronounced and as a consequence the selectivity in the low molecular weight region is increased. This effect was utilized for the separation of vasopressin and other peptides from amino acids by carrying out the gel filtration experiment in a solvent composed of acetic acid, pyridine and water (60: 15: 25 parts by volume) (Porath and Lindner, 1961). The same technique has also been used for the purification of hypophyseal melanophore stimulating hormones (Porath, personal communucation).

Many large peptides and small proteins have been purified with the aid of Sephadex G-50 and G-75. Thus, Gelotte and Krantz (1959) by two consecutive gel filtrations obtained a pepsin with a specific acitivity corresponding to 80 per cent of that of the crystalline enzyme from a crude commercial preparation. In a 2 litre column 37 grams were run in each experiment. To concentrate the pepsin, dry Sephadex G-25 (cf. page 55) was used, since the other methods tried gave appreciable losses of enzyme activity. Porath (1960) reported that lysozyme and cytochrome C were retarded in a column packed with an early laboratory made batch of dextran gel with slightly higher water regain than Sephadex G-50. In G-75 these enzymes are even more retarded and can be efficiently removed from material of larger size. An example of the purification of cytochrome C was given on page 61.

A detailed study of the behaviour of pancreatic enzymes has been made by Gelotte (1961; full details will be published later). In extracts made at pH 5, and passed through a Sephadex G-75 column at the same pH, the lipase activity appeared with the void volume (Fig. 16). The proteolytic activities gave a single peak and the ribonuclease activity appeared as a broad peak between the lipase and proteolytic enzyme peaks. This is contrary to the expected behaviour and is probably due to complex formation. Another anomaly was observed with amylase which appeared at a position which varied with the experimental conditions, e.g. the flow rate. This was interpreted as due to interaction between the gel matrix and the enzyme, which seems a reasonable hypothesis in view of the fact that the substrate for amylase has a structure similar to that of dextran. With acid (pH = 1) extracts of pancreas powder, the proteolytic and ribonuclease activities were eluted as after extraction at pH 5. In both types of extract

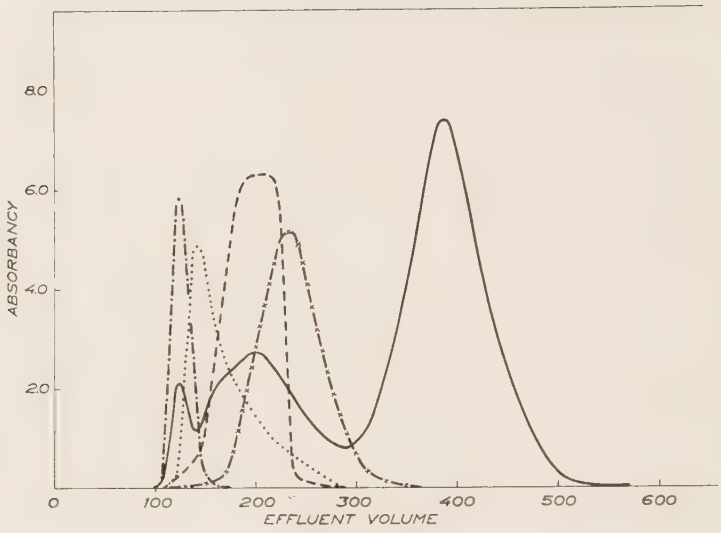


Fig. 16.

Elution diagram of pancreatic enzymes from a Sephadex G-75 column (4 cm × 30.6 cm). The absorbancy at 280 mμ (——) and the result of the assays for four enzymatic activites are shown. These were the lipase (.-.-), ribonuclease (....), proteolytic (- - -), and amylase (-×-×) activities. (Published by kind permission of Dr. B. Gelotte).

the main part of the UV-absorbing material was separated from the enzymes.

In Sephadex G-75 snake venom enzymes could be fractionated to a certain extent (Björk and Porath, 1959). Most enzymatic activities appeared with the void volume but the lecithinase A activity appeared as a separate peak ahead of the amino acids. By tryptic degradation of γ-globulin and gel filtration in Sephadex G-75 Hansson and Johansson (1960) were able to obtain four UV-absorbing peaks. Immuno-electrophoretic examination of the material from the first three peaks revealed that the components had differing immunological properties.

Two peaks with homocysteine methyl pherase activites were obtained by gel filtration on Sephadex G-75 (Klee et al., 1961). They differed in specificity and represented two different enzymes. Partially purified phosphatase from Escherichia coli could be separated from larger proteins in molar acetic acid. At high pH-values it migrated through a G-75 column without retardation (von Hofsten and Porath, 1961). In acetic acid solutions Crestfield has been able to separate the ribonuclease monomer and dimer (S. Moore, personal communication).

Some fractionations of proteins known to form aggregates have been reported. Thus, Cruft (1961) found on elution of ox thymus histones through Sephadex G-75 with 0.02 molar hydrochloric acid that α-histones were eluted

first. These are known to be of low molecular weight and are probably present in an aggregated state. In the second peak α- and β-histones were found and when rerun it appeared as two peaks indicating rather loose aggregates. The two slower peaks contained γ- and unaggregated β-histones, respectively. Cruft made the interesting observation that the Sephadex material did not deteriorate in contact with 0.02 molar HCl over a period of six months at room temperature. Bennich (personal communication) in studies of casein under a variety of conditions found elution peaks containing loose aggregates which separated when rerun. Under suitable conditions pure α- and β-caseins were obtained.

In a recent paper Rasmussen and Craig (1962) compared gel filtration and countercurrent distribution for the purification of parathyroid hormone. In an earlier publication (Rasmussen and Craig, 1961) only partial success with the gel filtration technique was reported, but with a more suitable electrolyte environment extremely efficient separations were obtained. With the 120—200 mesh sieve fraction of Sephadex G-50 and in the presence of 0.2 molar ammonium acetate a first elution in a column, 3.0 cm by 210 cm, and a subsequent rerun of the active fraction in a column, 0.9 cm by 150 cm, were sufficient to yield the hormone in pure form from a crude parathyroid extract. In a 1300 transfer countercurrent distribution experiment of the same crude material the

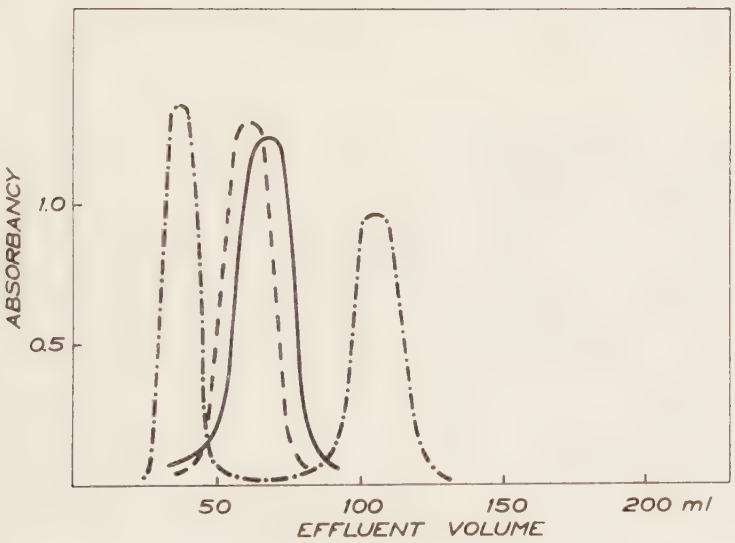


Fig. 17.

Elution patterns of serum albumin (.-.-.-), lysozyme (- - - -), and insulin (——) in a 2 cm × 36,5 cm, column, of Sephadex G-75. Eluant: 8 molar urea containing 0.1 molar phosphate buffer of pH 7.0. The last peak corresponds to a non-protein component present in the albumin preparation.

yield of active hormone was only 65 per cent of that obtained by gel filtration. The hormone obtained from countercurrent distribution invariably gave several peaks when subjected to gel filtration. In the presence of 0.2 molar acetic acid the parathyroid hormone was considerably more retarded on a Sephadex G-50 column than in ammonium acetate. Interpreted in terms of molecular sieving it means that the hormone had a smaller size in the presence of acetic acid. Support for this view was obtained by measurement of the rate of escape through a cellophane membrane. In 0.2 molar acetic acid the diffusion time was double that in 0.2 molar ammonium acetate.

Through gel filtration studies under selected conditions valuable information on the state of aggregation of protein mixtures can be obtained. It should also be possible to obtain information on the conformation of pure proteins by studying their behaviour in a column. Inspired by the finding of Craig et al. (1957) that the rate of escape of lysozyme through dialysis membranes was decreased in the presence of urea the author studied the same enzyme in Sephadex G-75. It was found that it was less retarded in the presence of 8 molar urea. Some experiments were also made with CO-hemoglobin with the hope that the urea-dissociated protein would be more retarded. This was not the case in 0.1 molar phosphate buffer at pH 7. On the other hand, insulin in the same buffer was retarded considerably more than lysozyme. In Fig. 17 are shown the elution curves of serum albumin, lysozyme and insulin, when run in the same column in the presence of 8 molar urea and 0.1 molar phosphate buffer.

Part 2. *In Sephadex G-100 and G-200*

Plasma proteins

The encouraging results of the fractionation of small proteins on Sephadex G-75 prompted trials with still more porous gel types. It soon became evident that highly porous gels made by the block polymerization technique were difficult to handle and gave inconveniently low flow rates. It was the development of bead polymerized gels (page 24), which made the use of more porous gels practical.

The fractionation of plasma proteins is of outstanding interest in many branches of science and though many excellent methods are available the complexity of the system makes new separation methods desirable. Fig. 18 shows the results of experiments with bovine plasma eluted under similar conditions on Sephadex G-75, G-100, and G-200. In G-75 there was only one peak but paper electrophoresis revealed that a partial separation had taken place with albumin enriched in the rear part of the peak. In G-100 two peaks were obtained and paper electrophoresis of the material in the fractions was performed. The second peak contained the albumin and a few minor com-

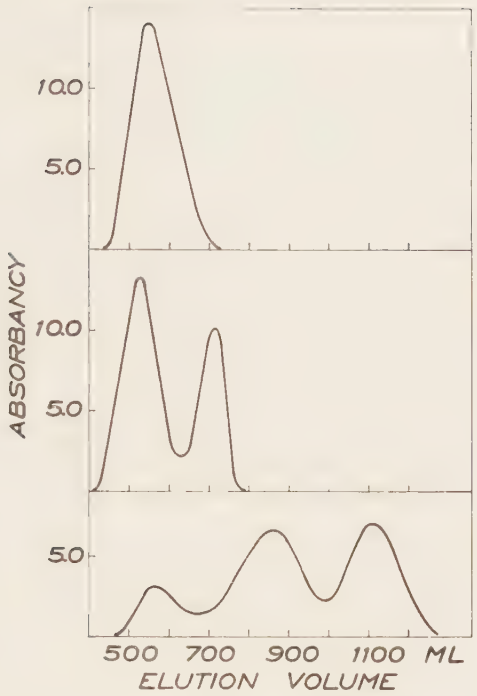


Fig. 18.

Elution patterns of bovine plasma in three Sephadex types. Top: G-75; center: G-100; bottom: G-200.

pounds, the most important of which was transferrin. Within the first peak of the G-100 experiment some separation had occured but was not large enough to split this peak. The slow proteins of the first G-100 peak were sufficiently retarded, however, in G-200 to form a separate peak, which contained γ-globulin as the main component (Flodin, 1962).

Analytical ultracentrifugation of the fractions from the G-200 experiment gave results which confirmed the expectation that a separation according to size had been attained. The fastest peak gave two major components with sedimentation coefficients of 18 S and 7 S, respectively. From evidence with human sera (Flodin and Killander, 1962) it is known that the 7 S component represents lipoproteins, which in spite of their large size sediment at a slow rate. The second peak gave a main component with 7 S sedimentation and the slowest peak seemed homogeneous in the ultracentrifuge and had a sedimentation coefficient of 4 S.

The first peak contained the majority of the lipids as evidenced by putting aliquots from each fraction on filter paper and dyeing with Sudan Black. Minor amounts were present in the second peak. In bovine sera the lipoproteins are visible due to the presence of carotine. They could also be visualized by dyeing

the serum before the gel filtration with Sudan Black as according to Ressler et al. (1961). Most of the black colour migrated with the first peak and only a minor amount with the second.

The experiences with Sephadex G-200 were so encouraging that a more extensive study was made with human sera in order to establish the distribution of some individual proteins. This work has been reported (Killander and Flodin, 1961; Flodin and Killander, 1962) and immunoelectrophoresis, immunodiffusion, density gradient ultracentrifugation, and antibody titrations were used in addition to analytical ultracentrifugation and paper electrophoresis to identify the proteins. As with bovine plasma three UV-absorbing peaks were obtained. The α_2-macroglobulin and β_2-macroglobulin were localized to the first peak. In this peak 19 S anti-A and cold agglutinin antibodies were located. The 7 S antibody fraction was entirely in the second peak and albumin, transferrin, and α_1-glycoprotein (3.5 S) in the third peak. Immunoelectrophoretic bands corresponding to α- and β-lipoproteins were present in the first peak and in paper electrophoresis two prealbumins were observed in the third peak. In one of the experiments hemoglobin was added. A haptoglobin-hemoglobin complex of the 2.1-type was identified in the first peak. Free hemoglobin appeared in the rear part of the third peak almost completely separated from albumin.

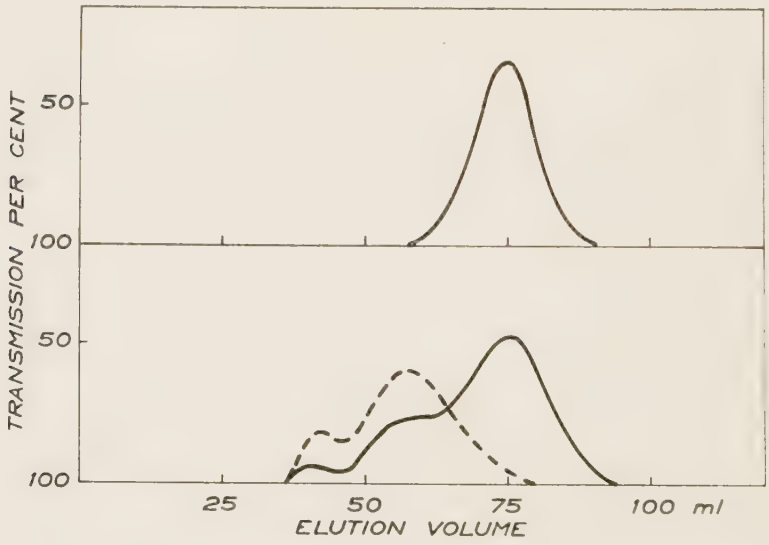


Fig. 19.

Rerun of the third peak from a serum separation in 0.1 molar phosphate pH 7.0 0.1 molar NaCl (upper diagram). Rerun of the second (- - -) and third (——) peaks from a serum separation in 0.1 molar phosphate buffer pH 7.0 (lower diagram).

Influence of pH and ionic strength. The pH *per se* does not seem to influence the separation and thus a pH at which the proteins have optimal stability can be chosen. In the first experiments made with G-200, 0.1 molar phosphate buffer of pH 7.0 was used and three peaks were obtained. When these peaks were rerun it became apparent that the separation had not been complete. The first peak again appeared homogeneous, the second was now bimodal with faster migrating proteins in the same portion as the first peak. The third peak was split into three components: the two fastest protein peaks corresponding to the proteins of the original first and second peaks. This overlapping was eliminated by working in a medium of higher ionic strength. A comparison between reruns of the γ-globulin and albumin peaks in 0.1 molar phosphate buffer at pH 7.0, with and without 0.1 molar sodium chloride, is shown in Fig. 19. The observation that the plasma proteins interact also when run in physiological saline solution may bear a relation to the state of the proteins in vivo.

Influence of column dimensions. Experiments have been made with equally good results in columns with diameters ranging from 2 to 7 cm and a bed height of 50 cm. By increasing the bed height, better resolution can be obtained due to the increased number of theoretical plates. No experiments were performed with

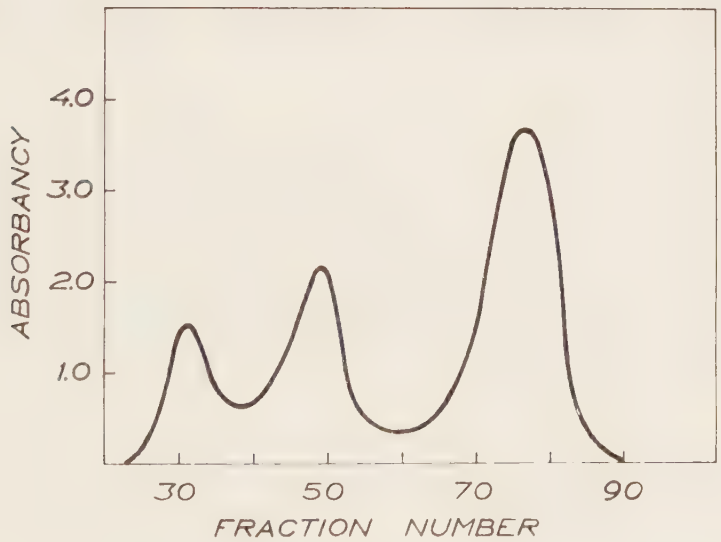


Fig. 20.

The elution diagram of human plasma (15 ml) from a column (4 cm × 132 cm) of a dextran gel with a water regain value of 15 g/g. Eluant: 0.1 molar tris-HCl + 1 molar NaCl. Elution rate: 20 ml per hour. Some fraction numbers are indicated in the diagram.

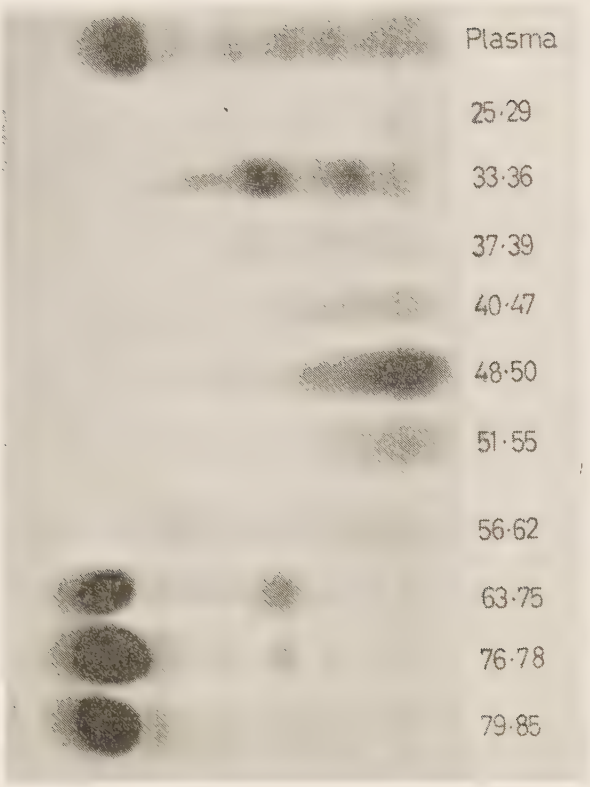


Fig. 21.

Paper electrophoresis diagram of the pooled plasma protein fractions from the experiment of Fig. 20 as indicated by numbers.

G-200 in longer columns, but with both G-100 (water regain 10) and an experimental batch with water regain 15 this proved to be the case. A human serum in a column of dimensions 4 cm by 132 cm yielded the elution pattern of Fig. 20. Analysis by paper electrophoresis showed that very little overlapping between the zones had occurred (Fig. 21). For example, the valley between the second and third peaks contained only minute amounts of albumin. Due to the lower water regain value compared with G-200 these peaks were displaced towards the first peak.

Influence of particle size. The size of the gel particle has a pronounced influence on the separation. This is illustrated in Fig. 8 B on page 39 where elution patterns of serum albumin on the 50—100, 100—200 and 200—270 mesh sieve fractions are shown. The finest fraction evidently gives the highest efficiency. When still finer fractions are used the flow rate is rapidly lowered and the experiment takes an excessive time. To obtain still sharper separations narrower sieve fractions should preferably be used and the 270—400 mesh fraction has given good results in the hands of the author.

Reproducibility. About one hundred separations of plasma proteins have been made in this laboratory and the reproducibility is surprisingly good. Many experiments have been performed in the same column without repacking. The primary cause of clogging with serum is the presence of lipoproteins. A high lipid content of the sera necessitates frequent repacking of the column. It has always been possible up to now, to regenerate the bed material by decantation.

Other proteins.

In spite of the short time the Sephadex types G-100 and G-200 have been available many interesting separations have been made. Thus, on Sephadex G-100 the lipase in pancreatic extracts was divided into two active components (Gelotte, personal communication). In a small column (1.5 cm by 38 cm), packed with the same material, a commercial cellulase from Aspergillus yielded four separate zones with cellulolytic activity and two zones with β-glucosidase activity (Pettersson, personal communication). α- and β-hydroxysteroid dehydrogenases, which are difficult to separate by other means, were completely separated in a single passage through Sephadex G-100 (Porath and Delin, personal communication). Some protein hormones have also been successfully fractionated, for example bovine thyrotropin (Porath and Condliffe, personal communication). In a column with the dimensions 4 cm by 75 cm human follicle stimulating hormone was separated from considerable quantities of inactive material (Roos, personal communication). With a column of Sephadex G-100 bovine growth hormone was purified considerably (Roos and Fevold, personal communication).

In columns of dimensions 4 cm by 150 cm packed with an experimental batch of bead polymerized dextran gel with water regain 15 g/g (corresponding to G-150) Pedersen (personal communication) has fractionated bovine serum albumin preparations. Four peaks were generally obtained and they contained albumin polymers, trimer, dimer, and monomer in the order of increasing retention. The two latter components could be obtained in ultracentrifugally homogenous form either directly or after a rerun. The procedure was highly reproducible as judged from more than twenty experiments.

CHAPTER 9

Adsorption and other side-effects

The ideal gel matrix for gel filtration should act only by virtue of its molecular sieve properties. No such system exists or is even likely to be found. In dextran gels many substances show a behaviour differing widely from what might be expected from their molecular size. These effects have been studied in detail by Gelotte (1960) who found evidence for at least two different mechanisms. The first is adsorption to the gel matrix and the second depends on the very few ionized groups present in the gels. It is possible, however, to utilize these effects for separation.

Adsorption.

The behaviour of a large number of water-soluble substances was studied by Gelotte (1960). It was notable that, of the inorganic substances studied, with sodium and potassium hydroxides and sodium tetraborate there was evidence of adsorption. This may be explained by the known ability of these substances to form complexes with polysaccharides. Of the amino acids phenylalanine, tyrosine, and tryptophane were adsorbed. The general conclusion was that aromatic and heterocyclic compounds had a larger tendency to be adsorbed than other water-soluble solutes. The adsorption of these was apparent both in distilled water and in the presence of salts. Porath (1960) studied amino acids and found the same type of behaviour. Of interest was the increase in adsorptivity obtained with the dinitrophenyl derivatives of amino acids. In molar pyridine, however, they moved faster than the parent amino acids.

The observed adsorption behaviour is in accordance with the affinity characteristics found for adsorption to cellulose (Giles, 1961). A planar structure and an extending system of conjugated bonds in the solutes favour adsorption and in view of the chemical similarity between dextran gels and cellulose similar mechanisms are probable. The observations of Lathe and Ruthven (1956) on the behaviour of many substances in starch columns support this view.

In many cases the adsorption isotherms have been found to be linear, and the solutes are therefore eluted as symmetrical peaks. Advantage can be taken of the adsorption side-effect which can be utilized to achieve separations for example of the aromatic amino acids (Porath, 1960; Gelotte, 1960). In Fig. 22 such a separation is illustrated. In many gel filtration experiments adsorption

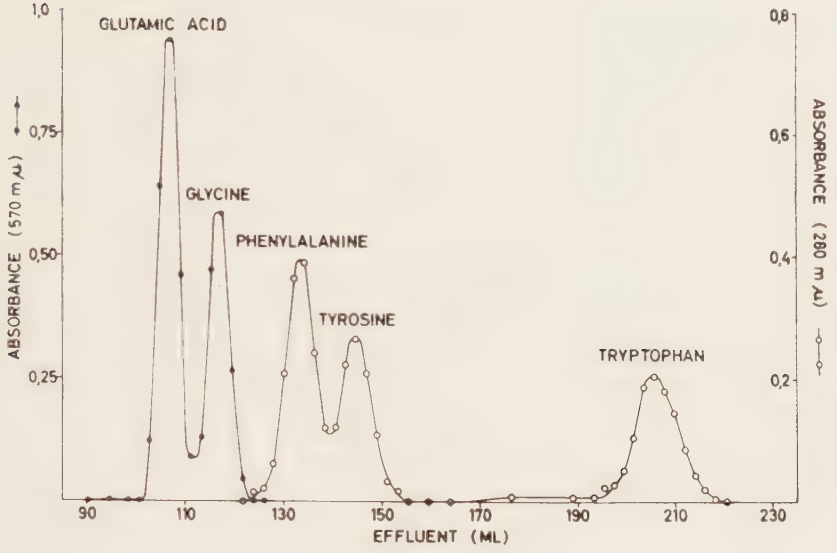


Fig. 22.

Elution pattern of a mixture of amino acids separated in Sephadex G-25 (200—400 mesh). Analysis of the fractions was made with the ninhydrin method (.-.-) and by UV-absorption (o-o-). The aromatic amino acids all had K_d-values higher than unity. (Reproduced by kind permission of Dr. B. Gelotte).

effects have produced a resolution not possible by molecular sieving. Thus nucleic acid components were resolved into many components some of which differed only slightly in size (Zadrazil et al., 1961; Gelotte, 1961).

Charge effects.

Many substances behave anomalously when subjected to gel filtration in distilled water. These effects are easily distinguished from adsorption in that they are normalized by the presence of salt. Basic amino acids, for example, are adsorbed, which can be explained by the presence of negatively charged fixed ions in the gel network (Gelotte, 1960; Porath, 1960). Evidence for the existence of such groups is also obtained by titration. Generally, amounts of 20—30 microequivalents are found per gram of dry gel.

The anomalous K_d-values obtained for many acids in distilled water probably also depend upon fixed charges. The acidic amino acids and nucleotides are almost completely excluded from the gel phase when eluted with distilled water (Gelotte, 1960). However, the presence of salt in the initial sample eliminates this effect, which is probably a kind of ion exclusion.

Combined adsorption and exclusion.

Very interesting effects are obtained when acids with a structure favourable for adsorption are passed through a Sephadex column. For example a pure dinitrophenyl aspartic acid is almost completely excluded when eluted with distilled water. In the presence of sodium chloride, however, it has a K_d-value higher than unity which indicates adsorption. When a sample containing dinitrophenyl aspartic acid and sodium chloride is eluted with distilled water the amino acid is concentrated during the passage and eluted in a volume smaller than that of the sample. This is explained in the following way: when the acid is in the salt zone it is adsorbed and thus retarded. The salt zone moves downwards and is followed by the water, which accelerates the acid due to exclusion. The final result is a concentration of the solute in the boundary between the salt and the water.

The described mechanism is probably responsible for the beautiful method for isolation of conjugated estrogens from urine described by Beling (1961). He used a column, 1 cm by 50 cm, with Sephadex G-25. Up to 20 ml of urine were added to the column and eluted with distilled water. The estrogens appeared in the effluent in only 3 ml and were recovered quantitatively. All the conjugated estrogens assayed for were present and most of the other materials in urine removed. In some experiments an additional estrogen peak was obtained, which had another composition. It is probable that many substances having a similar combination of properties can be purified in this way.

CHAPTER 10

Semi-continuous gel filtration

In gel filtration all solutes can be eluted without change of eluant. The bed is therefore regenerated during the experiment and a new separation can be started as soon as the slowest solute has left the column or even earlier (see below). This fact and the high reproducibility make an automatic operation of gel filtration columns possible.

In another separation technique, ion exclusion, the ability of ion exchange resins to discriminate between ionic and non-ionic solutes is utilized. No change of solvent is necessary and the process has been operated in a semi-continuous manner (Bauman et al., 1956). Even very large units have been used but so far no details of the construction of the equipment or the mode of operation was given.

The gel filtration unit.

A schematic drawing of a unit which has been used with bed dimensions from 10 cm by 50 cm to 20 cm by 100 cm, corresponding to bed volumes from 4 to 32 litres, is shown in Fig. 23. The main parts are the column (A), with accessories, the pumps (C1 and C2), and the timer (E). To meet the special requirements of automatic operation and the comparatively large size, some details, notably the outlet from the column and the distributor (B), deserve special attention.

The bed rests upon a 300 mesh stainless steel net which is supported by a perforated plastic plate with narrow drilled channels arranged in a symmetric pattern. The construction of the distributor (B) is shown in Fig. 24. The cylindrical device floats on the bed and thus follows any changes in the length of the gel bed and keeps the surface even. It is immersed in the column of eluant standing above the bed and the sample is applied at the interface between the gel and the eluant. A necessary condition is that the density of the sample is higher than that of the eluant. The holes (D) of the plastic arms of the distributor must be directed upwards in order to minimize convective flow during the resting periods. The diameter of the holes must be sufficiently large, so that the sample just flows over the edges, i.e. 1 mm in the separations reported below, because the formation of jet streams causes dilution. The eluant is pumped into the column through a perforated ring-shaped channel in the top of the column.

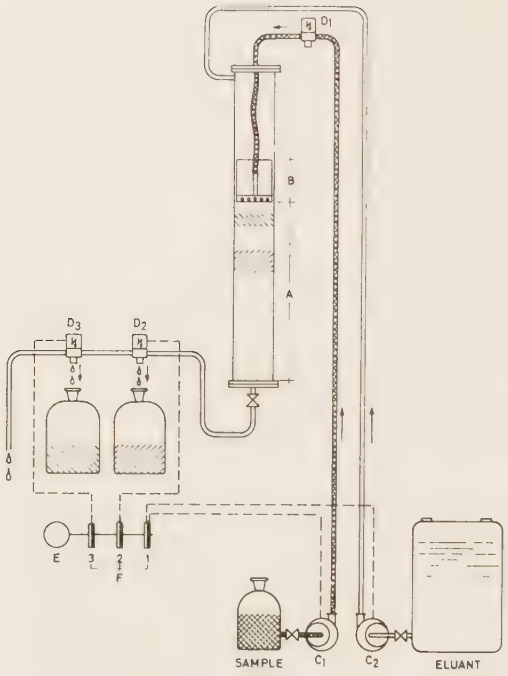


Fig. 23.

A schematic drawing of the semi-continuous filtration unit. The column A with the distributor B is fed with sample from the pump C 1, and with eluant from the pump C 2. D1 is a two-way solenoid valve closed when pump C 1 is not operating. D 2 and D 3 are three-way solenoid valves used for collection of fractions. The timer E with the cam pairs F 1, F 2, and F 3 regulates the pumps and the solenoid valves as indicated by broken lines.

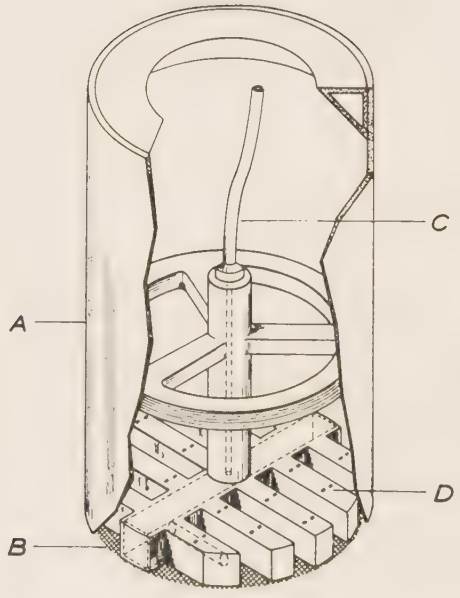


Fig. 24.

Drawing showing the construction of the distributor. A is a plastic cylinder with a stainless steel net B at the bottom. The sample is fed through the inlet tube C and layered over the bed through a plastic areas with holes D on the top side.

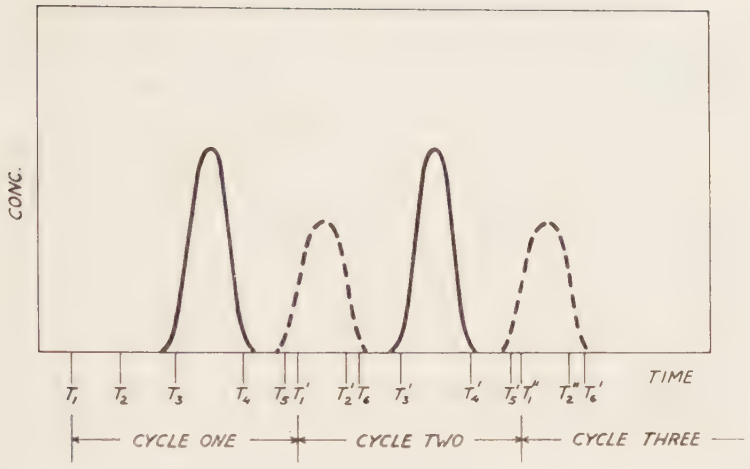


Fig. 25.

Elution curves from two cycles of a semi-continuous gel filtration experiment. The solid line represents the fast, and the broken line, the slow component. T_1, T_2 etc. indicate impulses from the timer as explained in the text.

The pumps (C1 and C2) of which C1 delivers the sample and C2 the eluant, are of a controlled volume type with adjustable flow rate. Membrane or plunge pumps can be used and to damp out disturbances due to their pulsations, partially airfilled flasks are placed in the tube lines between the pumps and the column.

The timer (E) is a conventional cyclic timer with a cam-shaft driven by a synchronous motor. By exchanging the gears the cycle time can be varied. Microswitches sliding over the cams transfer the information to the different parts of the apparatus. For the unit illustrated three pairs of cams are required. One pair (F1) operates the pumps and the two-way solenoid valve D1 and the other two (F2 and F3) operate the valves D2 and D3. By increasing the number of solenoid valves connected in series and the number of cams any desired number of fractions may be taken.

The sequence of the cycle. The principle for programming the gel filtration unit is illustrated in Fig. 25. At the time T_1 the sample pump starts to add sample to the column. When a suitable volume (time T_2) has been added the cam F1 switches off pump C1 and starts pump C2. Until time T_3 the effluent goes to waste and then the solenoid valve D2 is actuated through the cam F2 and the fast component collected. At T_4 the valve D2 is closed and the effluent goes to the waste until T_5 when the valve D3 is actuated by cam F3. D3 is open to time

T_6. In the meantime the cam-shaft has completed one revolution and the cam F1 starts the addition of a new sample. The time for the second and subsequent cycles is shorter than the time for a complete gel filtration experiment (first cycle) due to the fact that the void volume can be overlapped into a previous cycle.

In order to ascertain the program scheme a test elution curve is first made. For the first few cycles the bed changes in length but then reaches equilibrium, after which the program can be adjusted so that the unit operates without attention for long periods.

Factors influencing the performance.

Flow rate. The separation capacity depends on the cycle time which should be kept as low as possible. The possibility of keeping a high flow rate for long periods of time is therefore of decisive importance. The influence of the particle size of the Sephadex and the linear flow rate on the pressure drop over the column is illustrated in Fig. 26. It is evident that there is a limiting flow rate for each fraction at which the pressure increases very rapidly. With the 50—100 mesh fraction a flow rate of 1 cm minute is possible under practical conditions

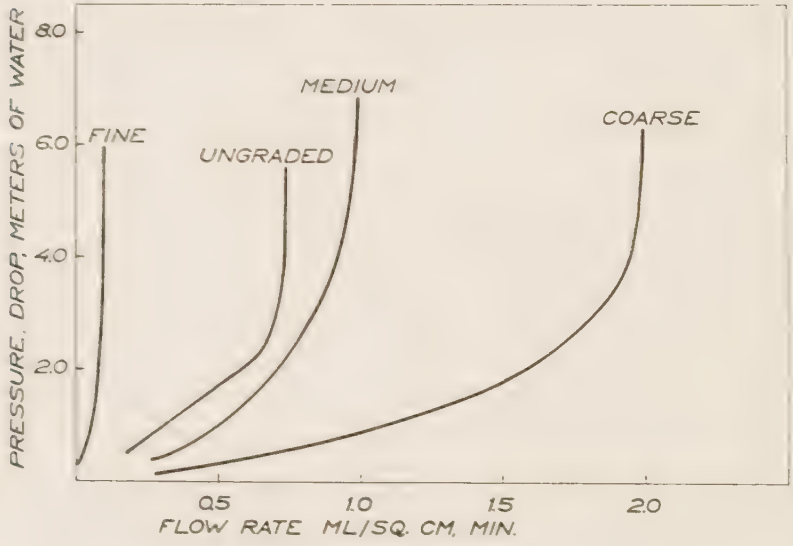


Fig. 26.

Diagram showing the relation between pressure drop and linear flow rate (ml cm^{-2} min^{-1} or cm min^{-1}) for 50 cm columns of Sephadex G-25. The particle size fractions, Coarse, Medium and Fine, indicated, correspond to the 50—100, 200—270, and 250—400 mesh sieve fractions, respectively. A curve for ungraded material is alsoshown.

and gives a cycle time of about 90 minutes for a 100 cm column. The more porous types of Sephadex are more elastic than the G-25 and therefore require lower flow rates and longer cycle times for corresponding particle size fractions. The length of the column is also of importance since the pressure drop increases with the length in a non-linear manner.

Particle size. As mentioned in chapter 4 the particle size has a profound influence on the efficiency of a column. It was concluded that particles as small as possible should be used. Only the 50—100 mesh fraction, however, gives such flow rate characteristics that reasonable separation capacities are obtained. It may be concluded that large capacities can only be obtained with separations requiring low column efficiency, such as desalting or crude fractionation.

Sample volume. In each cycle of a desalting process a sample volume corresponding to 20—30 per cent of the bed volume can be added without serious overlapping between the eluted zones. In order to keep the separation capacity high and the dilution of the zones low it it essential to add as large samples as possible.

Sample concentration. The same considerations as discussed on page 50 apply also to automatic gel filtration. Thus the concentration per se is relatively unimportant but high viscosity samples decrease the efficiency. When viscous zones pass through a column the resistance to flow increases and with it the pressure drop.

Applications.

The automatic semi-continuous process has been used mainly for three types of separation: the removal of salt from dextran and the removal of salt and simultaneous fractionation of the synthetic polymer "Ficoll", which is made by copolymerization of sucrose and epichlorohydrin (Flodin and Ingelman, to be published). Fractionations of low molecular weight dextrans have also been performed. Of these only the "Ficoll" experiments will be described here, since they were most thoroughly studied.

After polymerization "Ficoll" contains large amounts of salt, low molecular weight by-products, and low molecular weight polymers. In a cycling experiment a bed with the dimensions 20 cm by 100 cm packed with a 50—80 mesh sieve fraction of Sephadex G-25 was used. In each cycle a sample with an average volume of 4200 ml and containing 5 grams of Ficoll and 1.5 grams of sodium chloride per 100 ml was added. The flow rate was kept at 0.7—0.9 cm/min. and the time for each cycle was 2 hours. With regular intervals cycles were divided into small fractions and analyzed for optically active material and chloride ions. The diagram from cycle number 290 is shown in Fig. 27.

After 33 days and 398 cycles the pressure drop over the bed had increased to

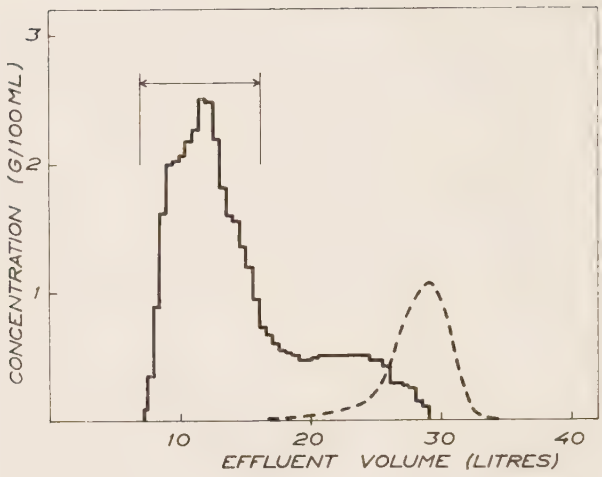


Fig. 27.

Elution pattern of cycle number 290 of a Ficoll fractionation. The thick solid line indicates optically active material. The broken line represents chloride ions. The arrows indicates the region in which the high molecular weight component was collected in the other cycles.

such a level that the experiment had to be interrupted. After a back-wash the column was again fit for use. The high molecular weight Ficoll was collected as indicated by arrows. The yield was 57 kg or 69 per cent, the dry Ficoll containing 0.4 per cent sodium chloride.

A large number of similar experiments have been performed under varying conditions. Concentrations of Ficoll up to 20 per cent have been separated but due to the high viscosity of the concentrated solutions the resolution was less good. With smaller bed dimensions good results were obtained and it was noted that the capacity per liter bed volume was higher with shorter beds. This depends on the non-linear pressure drop versus flow rate relationship referred to above.

Recently, preliminary experiment with bead-polymerized dextran gels have shown them to be much superior to the block polymerisates. For comparable particle size fractions and beds the former gave at least ten times higher flow rates with the same pressure drop. As a consequence, much shorter cycle times and also finer particle size fractions which increase the column efficiency can be used. It seems likely that the bead polymerisates will make possible the use of gel filtration on a large scale, at least for more costly products.

Summary

The purpose of this investigation has been to study the synthesis and properties of dextran gels and to develop methods to utilize their molecular sieve properties.

In the presence of alkali, aqueous solutions of polysaccharides and polyhydric alcohols can be cross-linked to gels by their reactions with diepoxides or substances reacting as diepoxides. The reaction of dextrans with epichlorohydrin under varying conditions is described. Widely differing porosities, measured as the water regain values, are obtained by varying the dextran and epichlorohydrin concentrations. High yields of porous gels are only obtained with dextrans of high molecular weight.

In the cross-linking reaction the dextran chains become connected by glyceryl bridges. The ether linkages thus formed are unreactive and the gels formed are therefore chemically stable. Due to the swelling of the network the cross-linking sites become loci for the closest approach of the dextran chains and therefore gradients which may be called "glucose residue density gradients" are formed. It is suggested that these are responsible for the molecular sieve action of the gels by causing restrictions to diffusion. With decreasing size of the solute molecules, larger solvent volumes within the gel become accessible for penetration.

In particulate form, the gels can thus be utilized for separations according to size. This is most conveniently performed with a chromatographic procedure, which has been called gel filtration. From studies with varying particle size of the gels and varying elution rates it was concluded that decreasing the particle size is the best way to improve the separation. The efficiencies under varying conditions, for commercially available types of dextran gels (Sephadex), are given in terms of the equivalent height of a theoretical plate (EHTP). Practical aspects of the treatment of the gels, and use of columns packed with them, are given together with methods for calculating distribution coefficients.

The use of dextran gels for separation is illustrated by a number of experiments made in this and other laboratories. The gels are very valuble for desalting and removing small molecular species from macromolecules. For this purpose rather inefficient columns can be used. For the separation of species with small differences in size, longer columns and smaler gel particle sizes must be used. This is illustrated in the separation of oligosaccharides and the fractionation of soluble dextrans.

For peptides and proteins the situation is more complicated due to protein-protein and protein-gel interactions. Many valuable separations on the basis of size alone can be achieved, however, if the effect of these interactions is minimized by working in a suitable medium. In the dextran gel types Sephadex G-25, G-50, and G-75 only species with molecular weights below about 50,000 can be fractionated. In the newer dextran gels, Sephadex G-100 and G-200, made by bead polymerization, even larger molecules are penetrant. Their use is exemplified by fractionations of plasma proteins, which can be separated into three groups with different sedimentation coefficients.

Many aromatic and heterocyclic substances are adsorbed to the gel matrix.

The gel filtration procedure can be performed in a semi-continuous manner. An automatic unit is described, with which large volumes of solutions can be fractionated as exemplified by the purification of the synthetic polymer "Ficoll".

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